



# THE JOURNAL OF NUTRITION

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# A CYTOCHEMICAL STUDY OF THE RESPONSES OF THE ADRENAL CORTEX OF THE RAT TO THIAMINE, RIBOFLAVIN, AND PYRIDOXINE DEFICIENCIES<sup>1,2</sup>

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FOURTEEN FIGURES

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The current concept of adrenal physiology is that at least 2 types of hormones are elaborated in the adrenal cortex. The first of these, which may be described in terms of its prototype desoxycorticosterone, regulates the concentration of electrolytes in the tissues and is apparently elaborated in the glomerular zone of the adrenal cortex. The second, the prototype of which is corticosterone, controls gluconeogenesis and appears to be secreted by the cells of the zona fasciculata. The first of these hormones does not seem to be regulated importantly by the anterior pituitary gland, whereas the second is (Swann, '40; Deane and Greep, '46). Therefore, the observation that pantothenic acid deficiency caused enlargement of the adrenal gland, accompanied by a reduction in the steroid content of the fasciculata and by a decrease in weight of the thymus (an index of pituitary-adrenal activity), suggests that an effect of the vitamin deficiency had been to stimulate an

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<sup>2</sup> We are indebted to Merck and Company, Inc., Rahway, N. J., for the crystalline vitamins used in these studies.

excessive secretion of corticosterone and its homologues (Deane and McKibbin, '46). Such a response is entirely comparable to the production of the adaptation syndrome of Selye ('46), in which physiological stress leads to a homeostatic increase in the activity of the pituitary and adrenal glands.

Since pantothenic acid deficiency thus alters the activity of the biological mechanism which regulates gluconeogenesis, it seemed possible that this alteration might be a reaction to a disturbance in carbohydrate metabolism. Therefore other disturbances in carbohydrate metabolism might cause the same effects. To test this possibility, deficiencies in thiamine and riboflavin, known to be constituents of coenzymes for the oxidation of carbohydrates, were studied as well as a deficiency of pyridoxine, believed to regulate transamination (Hawkins, MacFarland and McHenry, '46). After the first experiment had shown that thiamine deficiency rapidly activated the pituitary-adrenal-thymus system, the study was extended to determine whether the response was caused by the vitamin deficiency *per se* or by the partial inanition induced by the deficiency.

#### MATERIAL AND METHODS

For the first experiment, 20 young male rats of the Long-Evans strain were used. Seven controls were fed a purified diet (table 1) essentially similar to that employed by Deane and McKibbin, but with the addition of *p*-aminobenzoic acid, inositol and biotin in an attempt to prevent the fatty infiltration of the liver observed in the control animals of the previous investigation. Five rats were maintained on this purified ration lacking thiamine, 4 on the ration lacking riboflavin, and 4 on the ration lacking pyridoxine. Control and deficient animals were killed together at intervals according to the severity of the deficiency signs (table 2). The rats were killed in the forenoon, when glycogen is normally present in the liver and its quantity may be judged.

For the second experiment, 20 young male rats of the Long-Evans strain were distributed in groups of 4 according

to weight. Of each group, 1 was fed the thiamine deficient diet (TD) and the other 3 were fed the control purified diet. One of the controls was paired with the deficient rat so that its weight was similarly restricted (WR), and the second had its food intake restricted (IR) to that of the deficient. The third was permitted to eat *ad libitum* (AL). Groups were killed at the end of 1, 2 and 3 weeks.

TABLE 1  
*Constituents of complete purified diet.*

BASAL MIXTURE		VITAMINS AND OTHER SUPPLEMENTS			
Sucrose (gm)	67	Thiamine-HCl ( $\mu$ g)	250		
Casein, purified <sup>1</sup> (gm)	24	Pyridoxine-HCl ( $\mu$ g)	250	Choline chloride (mg)	100
Salt mixture <sup>2</sup> (gm)	4	Riboflavin ( $\mu$ g)	300	Inositol (mg)	100
Corn oil (gm)	5	Niacin (mg)	2.5	Irradiated ergosterol (I.U.)	310
		Ca-pantothenate (mg)	2	$\alpha$ -tocopherol (mg)	5
		<i>p</i> -aminobenzoic acid (mg)	30	$\beta$ -carotene (mg)	1.1
		Biotin ( $\mu$ g)	40	2-methyl,1,4-naphtho- quinone ( $\mu$ g)	600

<sup>1</sup> SMA.

<sup>2</sup> Phillips and Hart.

At autopsy the paired adrenals and the thymus of each animal were weighed on a Roller-Smith precision balance. The adrenals and pieces of liver were then fixed in 10% neutralized formalin for lipids and in Maximow's Zenker-formalin for mitochondria. In addition, in the first experiment, pieces of liver were fixed for glycogen in Rossman's picro-alcohol-formalin.

For the cytochemical demonstration of lipids, the formalin-fixed blocks were cut on a freezing microtome at 15  $\mu$ . From each block 1 section was stained with sudan IV and Harris' hematoxylin, another with sudan black B, and a third by the

Schiff plasmal method. A fourth section was mounted unstained, and a fifth was extracted with acetone before mounting. The latter sections were viewed on the polarizing and fluorescence microscopes for substances which are birefringent, possess a greenish-white autofluorescence, and are acetone-soluble. Any acetone-soluble, sudanophilic material which is also Schiff-positive, birefringent and autofluorescent may be considered a ketosteroid, since no other single class of substances gives all of these reactions (Dempsey and Wislocki, '46).

For mitochondrial preparations the Maximov-fixed blocks were postchromated for 3 days at 37°C., sectioned in paraffin at 3  $\mu$ , and stained with Mallory's phosphotungstic acid hematoxylin. To demonstrate liver glycogen, the pieces fixed in Rossman's fluid were sectioned in paraffin at 5  $\mu$  and stained by the Bauer-Feulgen technique (Bensley, '39).

#### OBSERVATIONS

##### *Deficiencies of thiamine, riboflavin, and pyridoxine*

*Weight changes.* Table 2 presents the changes in the weight of the rats and the proportional weights of their thymuses and paired adrenals in the first experiments. The controls grew steadily and well on the purified diet. The thiamine deficient rats ceased to gain weight by the end of the second week, were losing weight by the end of the third, and were below their initial weight and moribund after 4 weeks on the diet. The rats deficient in riboflavin and in pyridoxine continued to gain weight at a slow rate throughout the experimental period of 10 weeks, never becoming moribund. The only period of marked illness in these 2 groups occurred between the second and fourth weeks, after which there was some regression of the acuteness of the signs of their deficiencies.

In the control group, the weights of the paired adrenals and of the thymus gland drop gradually in relation to body weight, as is characteristic of normal ageing (Korenchevsky, '42).

In thiamine deficiency, on the other hand, a progressive increase in the relative weight of the adrenals occurs during the experimental period of 4½ weeks, and the atrophy of the thymus is far more rapid than normal. In the absence of

TABLE 2

*Data on rats fed diets deficient in thiamine, or riboflavin, or pyridoxine and their controls.*

DIET	ANIMAL	WEEKS ON DIET	INITIAL WT.	PEAK WT. <sup>1</sup>	TERM WT.	WT. CHANGE	ADRENAL PROP. <sup>2</sup>	THYMUS PROP. <sup>2</sup>
			gm	gm	gm	gm	mg/100 gm	
Control	A	3	62	140 (21d)	140	+ 78	16.2	260
	B	3	56	138 (21d)	138	+ 82	21.3	402
	C	4	56	166 (28d)	166	+ 110	23.5	332
	D	6	56	198 (42d)	198	+ 142	14.5	234
	E	8	74	258 (56d)	258	+ 184	15.1	236
	F	10	70	241 (70d)	241	+ 141	13.5	155
	G	10	49	229 (70d)	229	+ 180	14.2	92
Thiamine deficient	A	3	92	130 (14d)	112	+ 10	27.1	61
	B	3	94	135 (14d)				
	C	3	72	102 (14d)	72	0	29.8	124
	D	4	97	143 (14d)	93	— 4	38.6	27
	E	4½	102	139 (14d)	94	— 8	38.4	62
Riboflavin deficient	A	3	57	77 (14d)	65	+ 8	30.9	169
	B	6	57	76 (42d)	76	+ 19	26.0	47
	C	8	73	99 (56d)	99	+ 26	18.8	117
	D	10	73	133 (63d)	132	+ 59	16.8	148
Pyridoxine deficient	A	3	42	67 (21d)	67	+ 25	25.8	51
	B	6	44	83 (24d)	70	+ 26	32.6	42
	C	8	46	114 (56d)	114	+ 68	22.8	
	D	10	42	123 (70d)	123	+ 81	18.2	86

<sup>1</sup> Data in parentheses give day when their maximum weight was attained.

<sup>2</sup> Proportional weight.

riboflavin or pyridoxine from the diet, however, the adrenals remain relatively large up to 6 weeks and then become smaller. Riboflavin deficiency causes little change in the thymus gland, whereas pyridoxine deficiency results in considerable atrophy. Thus the 3 deficiencies studied produce entirely different patterns of response in the adrenals and thymus.



*Histological results. a. Controls rats.* In all controls, the adrenal cortex presents the normal picture. Four zones are distinguishable by the organization of the cells and by their lipid contents. The subcapsular zone, or glomerulosa, is composed of loops of cells filled with moderate sized, deeply staining sudanophilic droplets (fig. 1) which are, in addition, acetone soluble, Schiff-positive (fig. 4), autofluorescent, and birefringent (fig. 8). Here the birefringent particles are crowded and usually both coarse and fine in size. (The work of Weaver and Nelson ('43) indicates that small particles are in the process of being released from the cells, whereas the coarse ones are storage material.) There follows a narrow, fat-free transitional zone. Then comes the zona fasciculata, comprising long cords of cells separated by narrow sinusoids. On the basis of lipid content this zone may be divided into 2 sections — a broad, outer region in which the cells are swollen with small, pale sudanophilic droplets and a narrower, inner region in which lipids are greatly reduced in amount. The lipid droplets of the fasciculata contain ketosteroids as evidenced by the battery of histochemical tests. The birefringent material is sparser here than in the glomerulosa, especially in the younger animals, and consists of scattered coarse particles with many fine ones intermixed. The cords of cells are somewhat disorganized immediately adjacent to the medulla (zona reticularis), and the sinusoids anastomose into larger vessels. Here the cells contain sudanophilic droplets of irregular size which fail to display all of the ketosteroid reactions and probably may be considered to be triglycerides.

Mitochondrial preparations of the adrenals of these rats likewise present the normal picture. These organelles are fine granules in the glomerulosa, slightly larger spheres in the outer fasciculata, and again small granules in the inner fasciculata and reticularis (Deane and Greep, '46, figs. 22 and 23).

The livers of some of these rats exhibit some fatty infiltration but far less extremely than with the previous diet (Deane and McKibbin). Very little infiltration occurs by 6 weeks, but a moderate accumulation is variably present by 10 weeks. The

fat occurs in small droplets in the central cells of the lobule, but it fails to present the ketosteroid reactions observed in the former investigation. Apparently the addition of more vitamins to the purified diet partially rectifies the conditions leading to a fatty liver. This may be due principally to the inositol, which is known to possess lipotropic activity (Best et al., '46). A moderate amount of glycogen occurs in these livers and appears mostly in the peripheral part of the lobule. The hepatic cell mitochondria are small and exhibit the usual zonation by being spherical in the cells at the periphery of the lobule, rod-shaped in the intermediate zone, and fibrillar and very light staining in the center.

*b. Thiamine deficient rats.* The dietary lack of thiamine produces essentially the same alterations in the adrenal cortex of the rat that were observed in pantothenic acid deficiency. At 3 weeks the adrenal sections stained with sudans show an unchanged zona glomerulosa but a fasciculata containing less lipid than in the control preparations. Moreover, the fat-free transitional zone has disappeared. The Schiff reaction is intense in the glomerulosa but very faint in the fasciculata, where it is limited to the outer-most layers. A normal quantity of birefringent particles occurs in the glomerulosa, whereas in the fasciculata the birefringence is increased beyond the normal amount. Autofluorescence of usual intensity persists in the glomerulosa cells and in the outer half of the fasciculata.

At 4 and 4½ weeks, although the lipid of the glomerulosa is of almost normal quantity and retains all of the ketosteroid characteristics, sudanophilia has virtually disappeared from the fasciculata (fig. 3). Furthermore, the small amount of residual lipid does not possess the ketosteroid reactions.

Whereas the mitochondria in the cells of the zona glomerulosa in these adrenals are the usual small granules, those in the fasciculata differ distinctly from normal. They are greatly swollen, irregular in size within individual cells, and stain very slightly.

The liver preparations for these animals exhibit the following characteristics. Sudanophilic lipids are completely absent.

Glycogen is present in extremely small amounts. The phosphotungstic-acid hematoxylin preparations reveal that the hepatic cells are smaller than normal and the mitochondria are swollen, vesiculated and irregular in size.

*c. Riboflavin deficient rats.* The rats lacking riboflavin in the diet were killed at 3, 6, 8 and 10 weeks, respectively. The concentration and distribution of sudanophil lipids in the adrenals are within the usual range for all 4, and the ketosteroid tests are essentially normal, although the transitional zone has been obliterated (fig. 5). The quantity of birefringent particles is somewhat increased in the outer fasciculata. Paraffin sections of the adrenals, moreover, reveal that the fasciculata cells are smaller than normal but the mitochondria are spheres of the usual size.

The livers of the riboflavin deficient rats display a fatty infiltration at 3 and 6 weeks which is considerably greater than that which occurs in the corresponding control rats. Moreover, the fat is concentrated in the peripheral cells of the lobule and occurs in large-sized drops. It fails to give the ketosteroid reactions. By 8 and 10 weeks of deficiency, this fatty infiltration has disappeared. Glycogen is moderately concentrated in the liver cells and occurs chiefly near the central vein of the lobule. The mitochondria are normal.

*d. Pyridoxine deficient rats.* The 4 rats fed the pyridoxine deficient diet were killed at 3, 6, 8, and 10 weeks, respectively. Cytochemically their adrenal cortices indicate some stimulation at 3 weeks which disappears later. Thus at 3 weeks, although the glomerulosa appears normal, the fasciculata and reticularis display an augmented amount of sudanophilic lipids which fill all of the cells to the border of the medulla. This lipid is Schiff-positive and moderately autofluorescent, and the whole region contains crowded fine birefringent particles. At 6 and 10 weeks, however, the appearance of the lipid preparations is no different from that of the controls (fig. 6). The mitochondria are fine granules of normal appearance in all these adrenals.

The livers of the pyridoxine deficient rats are uniformly free of histologically demonstrable fat. They exhibit a moderate concentration of glycogen which is greater at the periphery of the lobule than at the center. The mitochondria are entirely normal.

*Comparison of reactions to thiamine deficiency and to inanition*

*Weight changes.* Five groups of 4 young male rats each were compared for the effect on the adrenal cortex and thymus of thiamine deficiency and comparable degree of inanition. Inspection of table 3 reveals that the deficient rats (TD) ceased gaining weight on about the eleventh day, the weight restricted (WR) rats on the twelfth, and the intake restricted rats (IR) about a day later. The *ad libitum* controls (AL) gained steadily and well. The adrenals of the deficient rats weigh proportionately most and their thymuses least; the weight restricted and intake restricted rats have large adrenals but larger thymuses; and the controls have the smallest adrenals and the largest thymuses. At 3 weeks the response seems to be most marked for group C, which comprised the youngest and smallest rats.

*Histological results.* *a. Control rats.* The 5 control rats (AL) all display normal adrenal cortices such as have been described above (figs. 7 and 8). Their livers contain only occasional fatty cells and present no other histological abnormalities.

*b. Thiamine deficient rats.* The rats fed the thiamine deficient diet (TD) show a progressive alteration of the adrenal cortex during the 3 weeks experimental period. The sequence of changes is unfortunately complicated by the fact that the larger animals are more resistant to the deficiency than are the smaller ones. Nevertheless, it appears to be as follows. By the end of 1 week (A) there is a slight reduction of sudanophil lipids in the fasciculata (fig. 2). By the end of 2 weeks (B), pituitary stimulation of the cortex is indicated not only by the increased weight of the adrenal and atrophy

of the thymus, but also by the augmentation of lipids in the fasciculata. Sudanophilic and birefringent material is increased in quantity and extends down to the border of the medulla (as in fig. 10). By 3 weeks the lipid content of the fasciculata cells is again reduced, more so in the smallest rat (C) than in the other 2 (D and E). In all 3 adrenals, lipids

TABLE 3  
*Data on thiamine deficient rats and their controls.*

EXP.	GROUP	WEEKS	INITIAL WT.	PEAK WT. <sup>1</sup>	TERM. WT.	WT. CHANGE	ADRENAL PROP. <sup>2</sup>	THYMUS PROP. <sup>2</sup>
			gm	gm	gm	gm	mg/100 gm	
Thiamine deficient (TD)	A	1	60	88 (7d)	88	+ 28	21.2	336
	B	2	75	99 (11d)	95	+ 20	25.0	192
	C	3	48	82 (9d)	60	+ 12	35.0	68
	D	3	57	86 (11d)	71	+ 14	26.6	100
	E <sup>3</sup>	3	82	117 (11d)	86	+ 4	26.5	44
			64.4	94.4	80	+ 15.6	26.9	148
Controls restricted in weight (WR)	A	1	68	94 (7d)	94	+ 26	18.9	330
	B	2	77	108 (7d)	105	+ 28	22.3	275
	C	3	53	90 (12d)	70	+ 17	28.4	73
	D	3	61	94 (12d)	76	+ 15	28.9	86
	E	3	85	129 (12d)	103	+ 18	26.1	159
			68.8	103	89.6	+ 20.8	25.1	185
Controls restricted in food intake (IR)	A	1	63	88 (7d)	88	+ 25	22.2	414
	B	2	74	105 (9d)	102	+ 28	26.0	302
	C	3	47	94 (14d)	66	+ 19	30.9	71
	D	3	51	87 (12d)	79	+ 28	27.0	215
	E	3	85	150 (14d)	102	+ 18	26.0	302
			64	104.8	87.4	+ 23.4	26.5	261
Controls fed <i>ad libitum</i> (AL)	A	1	65	89 (7d)	89	+ 24	19.8	279
	B	2	69	135 (14d)	135	+ 66	17.1	336
	C	3	51	152 (21d)	152	+ 101	15.4	270
	D	3	65	155 (21d)	155	+ 90	16.7	406
	E	3	82	141 (21d)	141	+ 59	17.2	184
			66.4	134.4	134.4	+ 68	17.2	295

<sup>1</sup> Data in parentheses indicate day when maximum weight was attained.

<sup>2</sup> Proportional weight.

<sup>3</sup> Wasted food, so that IR control is not valid.

which are sudanophilic (fig. 13), Schiff-positive and autofluorescent are limited to the outermost portion of the fasciculata. Furthermore, the quantity of birefringent material is subnormal in rat C but still about normal in D and E, although in the latter 2 there are virtually no fine particles (fig. 14).

The livers of the deficient rats show no fatty infiltration, and no signs of liver abnormality appear, except that the cells are smaller than normal.

*c. Weight restricted and intake restricted rats.* No essential differences occur between those rats that were paired with the deficient rats for weight change (WR) and those that were paired for food intake (IR). In addition to an adrenal hypertrophy like that of the deficient rats (but less atrophy of the thymus), the fasting rats possess adrenals that evince a similar but less extreme alteration in ketosteroid content which can be briefly summarized as follows. Throughout the experimental period the lipid of the glomerulosa remains unchanged in quantity and histochemical reaction. In the fasciculata, however, the sudanophilic lipids first rise above normal and then decline somewhat, especially in the small animals (figs. 9 and 11). The ketosteroids appear normal at the end of 1 week, increased in amount at 2 weeks, and greatly augmented the third week in D and E, though slightly reduced in C. The superabundant birefringent material in the fasciculatas of the larger animals at 3 weeks is illustrated in figures 10 and 12.

The livers of these 10 rats present no signs of any abnormality.

#### DISCUSSION

##### *Thiamine deficiency, inanition, and the adaptation syndrome*

Dietary deficiencies of 4 B-vitamins have now been studied with respect to their effect on the adrenal cortex and thymus of the rat. Two of these deficiencies, pantothenic acid and thiamine, cause a stimulation and ultimate exhaustion of the zona fasciculata of the adrenal cortex and a concomitant atrophy of the thymus. Stimulation of the adrenal cortex in

advanced thiamine deficiency has also been reported for the dog (Goodsell, '41a, b). In thiamine deficient rats the final exhaustion of the adrenal cortex may be gauged not only by the disappearance of the ketosteroid reactions from the zona fasciculata, but by such features as the abnormally swollen mitochondria in the fasciculata cells and the subnormal quantity of glycogen in the livers. We have found that the effect of thiamine deficiency is more rapid and more severe than that of a comparable degree of inanition, during which the adrenals are stimulated but do not become exhausted so quickly.

The reactions of the adrenals and thymus to these 2 B-vitamin deficiencies and to inanition fulfill the definition of the adaptation syndrome (Selye, '46). In this syndrome the production and release of the cortical steroids (probably those with an oxygen on C<sub>11</sub>) is stimulated by adrenotropin during physiological stress. These cortical hormones in turn increase protein catabolism and cause thymus involution (Dougherty and White, '45).

One consideration which emerges from the present study is the importance of the relative and progressive nature of the adrenal changes. These changes occur more rapidly with severe stress than they do with mild insults. Furthermore, stress of a given intensity seems to affect young animals more severely than it does older ones. Moderate stress leads to continued resistance, with an elevated content of adrenal steroids, and severe stress causes subsequent exhaustion of the adrenal. These stages of adrenal reactivity must be taken into account in evaluating experimental studies on the activity of the adrenal cortex. For example, in 1 such study, Foster et al. ('44) tested the resistance of thiamine deficient mice and pair-fed controls toward poliomyelitis. Between 10 and 20 days on the diets the deficient mice were more resistant to the disease than the inanition controls, and both were more resistant than *ad libitum* controls. Similarly Murray and Morgan ('46) observed an enhanced resistance to anoxia in guinea pigs fed a vitamin C deficient diet for 15 to 30 days in comparison to pair-fed controls. On the other hand, Smith, Oster

and Toman ('44) found thiamine deficient cats less resistant to low barometric pressure than starved controls. All such differences need to be related to the degree of adrenal stimulation. One study which has attempted to trace the course of adrenal changes in stress is that of Oleson and Bloor ('41). These investigators analyzed the adrenals of rabbits starved from 3 to more than 14 days. At 3 days the adrenal weights were below normal, although the steroid and triglyceride contents were elevated. Later the weight of the glands rose steadily and the percentage of steroid remained above normal while the triglyceride content fell. These chemical analyses provide further interest since they correlate with our observation that sudanophilia and the steroid reactions may change independently.

*Riboflavin and pyridoxine deficiencies.* In contrast to deficiencies of pantothenic acid or thiamine, the dietary absence of riboflavin or pyridoxine fails to produce the adaptation syndrome. Physiological tests have similarly failed to show adrenal stimulation in riboflavin deficiency (Wickson and Morgan, '46). Early in these 2 deficiencies a mild stimulation of the adrenal cortex occurs, but this later disappears. Possibly this transitory stimulation results from the moderate inanition induced in these animals, to which they later become adjusted. The time of stimulation coincides with that of the most acute signs of deficiency (p. 4).

In pyridoxine deficiency, however, even without signs of persisting adrenal activity, severe thymus atrophy occurs, as has also been reported by Stoerk and coworkers ('44, '46). Consequently thymus involution need not always be attributed to a high level of secretion of the corticosterone-like hormones. Perhaps this atrophy results from inadequate protein synthesis.

If the 4 deficiencies that have been studied were of similar severity, the hypothesis would seem to be invalid that the stimulus for the release of extra adrenotropin might be an upset in carbohydrate oxidation, since a riboflavin deficiency does not produce continuing adrenal stimulation. Therefore,



at present the most attractive hypothesis to explain the release of adrenotropin is that the lack of thiamine or pantothenic acid causes organ changes which increase the utilization of C<sub>11</sub>-oxygenated hormones (see, for example, Ralli, '46). If so, what such changes may be remains to be elucidated.

#### SUMMARY AND CONCLUSIONS

Severe dietary deficiencies of thiamine, riboflavin and pyridoxine were produced in weanling male rats. Weight changes of the adrenals and thymus glands, and cytochemical studies of the ketosteroids in the adrenal cortices show that the lack of thiamine causes stimulation of the zona fasciculata of the adrenal cortex in 2 weeks and its exhaustion in about 4 weeks in a fashion comparable to that induced by pantothenic acid deficiency (Deane and McKibbin, '46). These changes are attributed to a stimulation of the adrenal cortex by adrenotropin, resulting in an increased production and secretion of the corticosterone-like hormones. The latter hormones in turn produce thymus involution. In a paired feeding experiment it was found that stimulation of the fasciculata occurs earlier in thiamine deficiency than with a comparable degree of inanition.

On the other hand, neither a riboflavin nor a pyridoxine deficiency causes more than a transitory stimulation of the adrenal cortex, although a lack of pyridoxine results in acute involution of the thymus.

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Photomicrographs of adrenal cortices from glands fixed in 10% neutralized formalin for 2 days or more, then sectioned on the freezing microtome at 15  $\mu$ . Sections mounted in glycerin jelly

## PLATE 1

### EXPLANATION OF FIGURES

All figures  $\times 135$  Demarcation between cortex and medulla of adrenal drawn in

1-3 Stained with sudan IV and Harris' hematoxylin. Photographed with blue filter (Wratten H) to accentuate red stain.

1 First experiment; control rat, D, fed the control purified diet for 6 weeks. This figure shows the usual distribution of sudanophilic lipids in the adrenal cortex: rich in the cells of the zona glomerulosa, absent from the transitional zone, rich again in the outer part of the zona fasciculata, dwindling in the inner fasciculata, and variably present in the cells of the juxtamedullary zona reticularis.

2 Second experiment, thiamine deficient rat, A, fed the deficient diet for 1 week. The glomerulosa appears essentially normal; the outer fasciculata cells have watery vacuoles, and the whole fasciculata appears somewhat depleted of lipids.

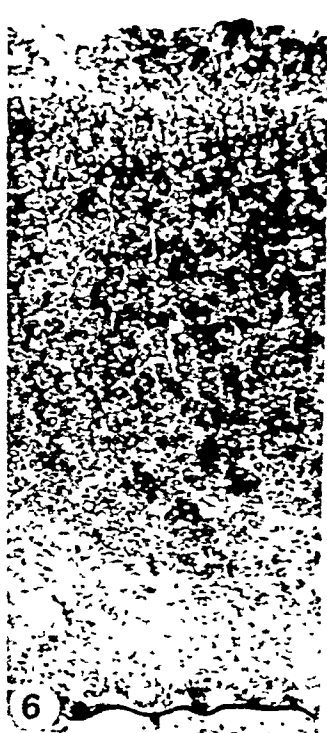
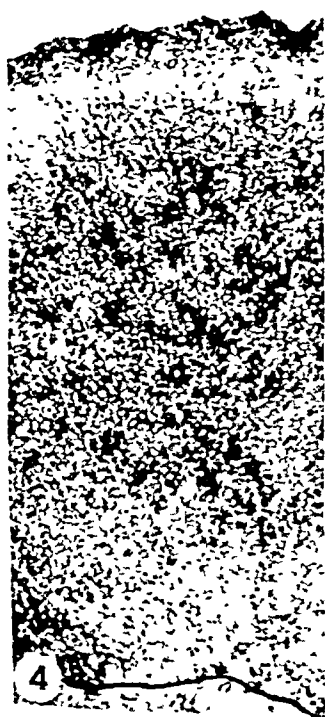
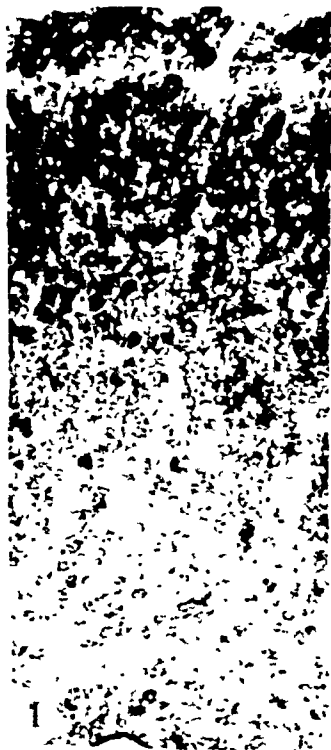
3 First experiment, thiamine deficient rat, D, fed the deficient diet for 4½ weeks. The animal was moribund when sacrificed. The entire fasciculata is exhausted of lipids, although the glomerulosa contains its usual complement. The cells of the outer fasciculata appear "vacuolated." The reticular arrangement of the cell cords appears to be extended outward.

4-6 Stained by Schiff plasmal method. Photographed with combined green and yellow filters (Wratten B and G) to accentuate the fuchsin stain.

4 Second experiment; *ad libitum* rat, C, fed the control, purified diet for 3 weeks. The Schiff positive material is normally distributed in the same fashion as is that of the sudanophilic lipids.

5 First experiment, riboflavin deficient rat, B, fed the deficient diet for 6 weeks. The usual quantity of Schiff-positive material is present in the cortex, although the transitional zone has been obliterated.

6 First experiment, pyridoxine deficient rat, C, fed the deficient diet for 8 weeks. The distribution of Schiff positive droplets is entirely normal.





# FURTHER STUDIES OF MANGANESE DEFICIENCY IN THE RABBIT

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In a previous paper from this laboratory (Smith, Medlicott and Ellis, '44), a number of differences between control and manganese deficient rabbits were described. The diets were fed ad libitum and some of the differences noted might well be ascribed to a difference in food intake, particularly since the growth rate of the control group was appreciably greater than that of the experimental group. By equalizing feed intake, those differences due specifically to a lack of manganese can be more readily differentiated from those due to a lack of other factors. The paired-feeding technique (Mitchell and Beadles, '30) was therefore used in the present study. A miscellany of results of several other manganese deficiency experiments which have not been reported elsewhere are also presented.

## EXPERIMENTAL

Dutch rabbits were bred to furnish young for the experiment. To prevent access to feed high in manganese, the stock diet was replaced by milk and milk powder when the young were 2 weeks of age, and the dams moved to another cage for 2 hours each day in which they were fed a stock diet of mixed grains and hay. The young were weaned at 3 weeks of age and placed on the experiment proper. Twelve pairs were selected, each consisting of litter mates of the same sex and

of nearly the same body weight. The rabbits were kept in individual wire-screened cages and fed a basal low-manganese diet of milk plus 10% of whole milk powder. Sufficient iron and copper were added to the diet to give a mean daily intake of 4 mg of iron and 0.4 mg of copper per animal. These supplements were fed twice weekly as the chlorides, prepared from redistilled  $\text{HCl}$  and carbonyl iron and electrolytic copper. One member of each pair was also given 2 mg of manganese (a solution of c.p.  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ) per day by mouth. The animals were weighed weekly until 17 weeks of age, at which time they were autopsied. Various tissues were taken as indicated below.

Three rabbits died before completion of the experiment. Two of these were a pair, while the third was on the low-manganese diet. Thus 10 pairs of rabbits were available for observation at the end of the experiment. The calcium and phosphorus content of the bone ash was determined by the methods of Morris, Nelson and Palmer ('31). The methods used to determine hemoglobin, liver manganese, and the ash, density, and breaking strength of the bones have been described (Smith, Medlicott and Ellis, '44). The inorganic phosphorus content of blood serum was determined by the method of Fiske and Subbarow ('25) using a photoelectric colorimeter. The "alkaline" phosphatases were determined by the King and Armstrong ('34) method at a pH of 9.8 (sodium barbital buffer), while the "acid" phosphatases were determined by the Gutman and Gutman ('40) procedure at a pH of 5.3 for all tissues except blood serum, which was run at a pH of 4.9. Tyrosine was used as the primary standard as recommended by Greenberg et al. ('40), but the results are expressed in terms of phenol, each unit being the amount of enzyme that liberates 1 mg of phenol in 1 hour under the prescribed conditions. The arginase values were obtained by a modified combination of the Edlbacher and Röthler ('25), and Lightbody ('38) methods as described by Kochakian ('44). Incubation was for a period of 4 hours at  $37^\circ\text{C}$ . and at a pH of 9.2. A unit is defined as that amount of enzyme pro-

ducing 1 millimol of urea in 4 hours under the prescribed conditions.

Redistilled water was used in the preparation of the substrates for both the arginase and phosphatase determinations as well as in the preparation of the tissue extracts. The soft tissues were homogenized according to the method of Potter and Elvehjem ('36), while the bones were ground with sand and water in a glass mortar and pestle. Where the bone ends and shafts were run separately, the division was made according to length, the 2 end quarters being combined. Three per cent of toluene by volume was added to the unfiltered extract, which was stored in the coldroom for 12-16 hours before an aliquot was removed for the determination.

The manganese determinations on blood serum and on the bones were made by the method of Gates and Ellis ('47).

The testes were fixed in Susa's fluid, imbedded in paraffin, sectioned at 8  $\mu$ , and stained with iron hematoxylin and eosin.

#### RESULTS OF THE PAIRED-FEEDING TRIAL

The rabbits fed the manganese-low milk diet showed the same gross symptoms previously described (Smith, Medlicott and Ellis, '44) whereas the rabbits fed the additional supplement of manganese were normal. Six of the 10 rabbits fed the milk diet showed gross crookedness of the front legs, which was confirmed by observation of the dissected bones. As in previous experiments, the bending was confined to the radius and ulna.

A comparison of the growth of control rabbits with those on the manganese-deficient diet is given in table 1. Though the food intake of the 2 groups was held constant, the control rabbits gained weight at a significantly higher rate than those fed the deficient diet, showing that a deficiency of manganese per se retards growth.

A summary of the studies on the bones is presented in table 2. The fresh weight, percentage of ash, total ash, density, and length of the humeri of the manganese-deficient rabbits were significantly less than those of the controls. The volume



TABLE 1  
*Growth of pair-fed control and manganese-deficient rabbits.*

TREATMENT	NO.	AGE AT AUTOPSY	MEAN DAILY FEED INTAKE	MEAN INITIAL WEIGHT	MEAN FINAL WEIGHT
		<i>wks.</i>	<i>ml</i>	<i>gm</i>	<i>gm</i>
Control	5 <sup>1</sup>	17	58.6	282	1240 <sup>2</sup>
Mn deficient	5	17	58.2	279	1053 <sup>2</sup>

<sup>1</sup> By error final weights obtained on only 5 of the 10 pairs.

<sup>2</sup> Difference significant at odds of 19:1.

TABLE 2  
*Tissue composition of pair-fed control and manganese-deficient rabbits.*

	CONTROL <sup>1</sup>	MN DEFICIENT <sup>1</sup>	MEAN DIFF	AND	S.E.
<i>Humerus</i>					
Fresh wt. (gm)	1.954	1.809	0.145	±	.0391 <sup>2</sup>
Ash (% dry fat-free bone)	58.88	57.03	1.85	±	.714 <sup>2</sup>
Ash (total)	0.5648	0.4702	0.0946	±	.02154 <sup>2</sup>
Volume (ml)	1.479	1.409	0.070	±	.034
Density (gm/ml)	1.318	1.282	0.036	±	.0148 <sup>2</sup>
Length (mm)	51.1	45.6	5.5	±	1.22 <sup>2</sup>
Breaking strength (lbs)	32.3	33.3	— 1.0	±	1.20
Ca (% dry fat-free bone)	22.47	21.95	0.52	±	.286
P (% dry fat-free bone)	10.67	10.22	0.45	±	.109 <sup>2</sup>
<i>Ulna</i>					
Fresh wt. (gm)	0.766	0.725	0.041	±	.0274
"Alkaline" Phosphatase (units/gm fresh wt.)					
No activation	25.9	12.7	13.2	±	1.36 <sup>2</sup>
Activation with Mn	30.9	15.0	15.9	±	1.19 <sup>2</sup>
<i>Femur</i>					
Fresh wt. (gm)	3.804	3.433	0.371	±	.0827 <sup>2</sup>
Dry fat-free wt. (gm)	1.934	1.592	0.342	±	.0558 <sup>2</sup>
Dry fat-free wt (% fresh bone)	50.3	45.8	4.5	±	1.0 <sup>2</sup>
Mn (μg/gm dry fat-free bone)	6.98	0.52	6.46	±	1.136 <sup>2</sup>
<i>Liver</i>					
Dry matter (%)	32.2	29.9	2.3	±	.91 <sup>2</sup>
Mn (μg/gm—dry basis)	6.04	0.43	5.61	±	.793 <sup>2</sup>
Arginase (units/gm fresh tissue)					
No activation	20.5	15.4	5.1	±	1.95 <sup>2</sup>
Activation with Mn	25.6	19.2	6.4	±	2.73 <sup>2</sup>
<i>Blood Serum</i>					
Mn (μg/100 ml)	4.8	2.0	2.8	±	1.57

<sup>1</sup> Mean values of 10 rabbits.

<sup>2</sup> Difference significant at odds of 99:1.

<sup>3</sup> Difference significant at odds of 19:1.

of the humeri of the 2 groups did not differ significantly. These observations confirm those previously found in experiments where food intake was not controlled. The breaking strength of the humeri of the 2 groups was found not to be significantly different, which varies from previous observations in which the diets were fed ad libitum. Further studies have indicated that the breaking strength of the bones of rabbits in these experiments is closely correlated with body weight. In another experiment (Smith and Ellis, '47), rabbits were fed no manganese, 0.3, 0.6, 1, or 8 mg of manganese per day. Within each treatment group the correlation coefficients between the breaking strength of the humeri and body weight were respectively, 0.73, 0.78, 0.69, 0.24, and 0.83. It thus appears that the breaking strength of bone is more closely associated with growth than with a deficiency of manganese per se.

Phosphorus and calcium determinations were made on the ash obtained from the humeri. No significant difference was observed for calcium, but the percentage of phosphorus in the dry, fat-free bones of the manganese-deficient rabbits was slightly but significantly lower than the controls. The meaning of this is obscure.

In this experiment the only tissue for which phosphatase values were obtained was the ulna. In one case the substrate used in the determination was made  $0.00004\text{ M}$  with respect to  $\text{Mn}^{++}$ , while in the other no Mn was added. In both cases, the "alkaline" phosphatase activity was approximately one-half as great in the deficient as in the control group.

The fresh and the dry fat-free weight of the femur were both greater in the control group, and this was also true of the percentage of dry fat-free bone. The manganese content of the femurs was approximately 13 times higher in the group given manganese than in the group not receiving it.

The dry matter, manganese content and arginase activity of the liver of the deficient group were all lower than in the controls. As in the phosphatase determination, the arginase activity was determined both with and without activation by

Mn. In this case the substrate was made 0.0004 M with respect to  $\text{Mn}^{++}$ .

In contrast to the marked reduction in the manganese content of the bone and liver of animals on the low-manganese diet, the reduction in serum manganese from 4.8 to 2 mg/100 ml was not significant at odds of 20 to 1. This is in agreement with the findings of Johnson ('40) that the level of manganese intake does not affect the blood values in either sheep or pigs.

#### RESULTS OF OTHER EXPERIMENTS

Phosphatase determinations were made on various tissues obtained from rabbits on 2 experiments in both of which the milk diet with and without a supplement of manganese was fed ad libitum. In one experiment the manganese was fed at 0, 1, 2, and 4 mg per day levels (Smith and Ellis, '47), and in the other 0 and 4 mg per day. There was no significant difference in either "acid" or "alkaline" phosphatase activity of the blood serum, kidney, liver, small intestine, and none in the "acid" phosphatase of the ulna. There was a significant difference in the "acid" phosphatase of the serum in one experiment, but this was not confirmed by the second experiment. The results are summarized in table 3. The number of animals is rather small to demonstrate conclusively that there are no differences, but it is clear that differences, if they exist, are much smaller than for the "alkaline" phosphatase activity of the bones.

Inorganic phosphorus determinations were made on the blood serum of the 0, 1, 2, and 4 mg per day rabbits with negative results as shown in table 3.

In previous work (Smith et al., '44) no change in the hemoglobin level of manganese-deficient rabbits was found, and this observation has been confirmed with larger numbers. In 62 rabbits (Smith and Ellis, '47) fed either the manganese-deficient diet alone or supplemented with 1, 2, or 4 mg of manganese per rabbit per day, the mean range of hemoglobin

TABLE 3  
Phosphatase values for various tissues of manganese-deficient  
and of control animals.

	DAYS ON FEED MG/DAY						
	0	1	2	3	4	5	6
No. of Animals	10	9	9	8	8	8	7
Inorganic P Serum (mg/100 ml)	7.36 ± .56 <sup>1</sup>	9.26 ± .84	8.41 ± .42	6.65 ± .99			
Phosphatase							
Blood Serum							
(units/100 ml)							
“Acid”	10.2 ± 1.24	14.9 ± .88	14.7 ± .99	14.4 ± .71	11.7 ± 1.43		12.1 ± .81
“Alkaline”	24.4 ± 1.80	25.2 ± 3.44	25.7 ± 2.90	24.3 ± 1.92	25.8 ± 2.38		25.1 ± 1.12
No. of Animals	6	4	9	3	4		1
Kidney <sup>2</sup>							
“Acid”	8.2 ± 1.00	8.6 ± .31	9.4 ± .55	9.6 ± .61			
“Alkaline”	49 ± 5.0	61 ± 14.6	65 ± 7.2	83 ± 23.4	48 ± 10.4		65 ± 10.1
Liver <sup>2</sup>							
“Acid”	6.2 ± 1.48	6.1 ± .16	6.7 ± .41	6.9 ± .60			
“Alkaline”	27 ± 5.6	19 ± 3.5	17 ± 1.7	19 ± .8	14 ± 3.9		16 ± 3.4
Small Intestine <sup>2</sup>							
“Acid”	5.4 ± .89	8.2 ± .74	8.6 ± .34	8.1 ± .08			
“Alkaline”	60 ± 18	101 ± 22	95 ± 11	104 ± 14	71 ± 19.0		42 ± 14.1
Bone End <sup>2</sup> (ulna)							
“Acid”	2.6 ± .61	2.9 ± .75	1.7 ± .47	1.8 ± .42			
“Alkaline”	9.3 ± 1.49	32.5 ± 2.62	27.8 ± 3.49	22.9 ± 2.95	19.6 ± 3.7		32.7 ± 6.9

<sup>1</sup> Standard error. <sup>2</sup> Units/gm fresh tissue.

values per treatment group was 10.47–11.22 gm per 100 ml of blood. These differences are not significant.

The effect of a deficiency of manganese on the testes of rabbits has been more thoroughly studied. Two out of 9 male rabbits fed the manganese-deficient diet showed a mild tubular degeneration after being fed the deficient diet for 12 to 22 weeks. Two of 12 comparable males fed manganese supplements (1, 2, or 4 mg per day) in addition to the basal diet also showed mild tubular degeneration. These observations indicate that a deficiency of manganese does not lead to testicular degeneration in the rabbit as it apparently does in the rat (Shils and McCollum, '43; Boyer, Shaw and Phillips, '42).

#### DISCUSSION

The rat (Shils and McCollum, '43; Boyer, Shaw and Phillips, '42) and the rabbit show a similar decrease in liver arginase activity both with and without manganese activation on a low-manganese diet when arginine monohydrochloride is used as the substrate in the arginase determination. Shils and McCollum found that when arginine carbonate was used as the substrate no difference in enzyme activity between manganese-deficient and control rats was found when manganese was used to activate the system, although there was a marked difference when no manganese was added. In view of this finding it is not possible to conclude that in manganese deficiency in the rabbit there is a reduction of liver arginase concentration.

The activation of "alkaline" phosphatase of the chick bone noted by Wiese et al. ('39) was confirmed both for the "acid" and "alkaline" phosphatase of the serum and for the "alkaline" phosphatase of the bone in the rabbit. These determinations were made both with and without manganese activation as an aid in the interpretation of the results. Since the differences noted occurred in both instances, the evidence suggests that the loss of enzyme activity is not due to a lowered concentration of manganese in the bone per se. This evidence, however, cannot be taken as conclusive since

manganese added in the determination may not activate the enzyme to the same extent as manganese found *in vivo*.

In making an interspecies comparison of the effects of manganese deficiency, it is interesting to note that in the chick there is a reduced "alkaline" phosphatase activity of the tibia and of the blood serum (Wiese et al., '39; Combs, Norris and Heuser, '42), while in the rat Amdur, Norris and Heuser ('45) found a small, but highly significant reduction of the "alkaline" phosphatase of the bones in manganese deficiency contrary to the findings of Wachtel, Elvehjem and Hart ('43). In the rabbit, the "alkaline" phosphatase of the bone is reduced, as in the chick and rat.

There is growing evidence also that the skeletal development is impaired in the rat and mouse (Shils and McCollum, '43; Amdur, Norris and Heuser, '45; Barnes, Sperling and Maynard, '41; Wachtel, Elvehjem and Hart, '43), in the chick (Caskey, Gallup and Norris, '39), and the rabbit (Smith, Medlicott and Ellis, '44), and is suggested by the results of Miller, Keith, McCarty and Thorp ('40) with swine. Thus in the rat, chick, and rabbit bone changes have been shown to be associated with a decreased "alkaline" phosphatase activity of the bones in manganese deficiency, and it seems clear that manganese is concerned specifically in bone formation probably through its influence on phosphatase activity.

Likewise, growth has been shown to be retarded in the manganese-deficient chick, rat, mouse, and rabbit by these workers.

Other symptoms of manganese deficiency, such as testicular degeneration, impaired lactation, and nervous disorders have been found less consistently and may reflect a varying severity of the deficiency.

#### SUMMARY

Manganese deficiency in the rabbit was studied using the paired-feeding technique with a basal diet of milk.

On the unsupplemented diet there was a lowered arginase activity of the liver and a lowered "alkaline" phosphatase activity of the ulna. The latter finding is associated with

impaired bone formation noted in the gross as crooked front legs. The fresh weight, percentage ash, total ash, density and length of the humerus was significantly lower in the manganese-deficient animals.

Though the food intake of the 2 groups was equalized, the controls gained more weight than those fed the low-manganese diet.

In the manganese-deficient animals the concentration of manganese in the liver and in the femur was much reduced, while that in the blood serum was not.

In other experiments in which the feed intake was not equalized, no significant differences were found for the following: "acid" or "alkaline" phosphatase activity of the blood serum, kidney, liver, or small intestine; hemoglobin concentration of the blood; inorganic phosphorus of the serum; incidence of testicular degeneration.

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# STUDIES OF THE MANGANESE REQUIREMENT OF RABBITS

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The manganese requirement has been established for poultry (Wilgus, Norris and Heuser, '39), but for no other animal insofar as the authors are aware. Following the background studies which characterized the symptoms of manganese deficiency in the rabbit (Smith, Medlicott and Ellis, '44), it became possible to determine the minimum amount of manganese required to prevent these symptoms. This paper summarizes the results of such studies.

Knowledge of the requirement of any nutrient for various metabolic processes is essential as a baseline for assessing the adequacy of a feed or ration for that nutrient. It is realized that any suggested value is subject to variation dependent on many factors. Among such factors are the availability of the nutrient which is a function of the chemical form in which it is fed as well as of the effects of other components of the diet. The requirement is also subject to genetic variability as is often noted in breed or strain differences, the age of the animals, and the criteria used to determine the adequacy of the nutrient levels fed. The established deficiency of a milk diet with respect to manganese, the general adequacy of a milk diet in nutrients other than manganese, iron, and copper, and a lack of knowledge of a

practical ration of forages and concentrates sufficiently low in manganese to serve as a basal diet prompted the use of milk and milk products in this study.

#### MATERIALS AND METHODS

The technics and materials used in this study have been outlined in detail in a previous publication (Smith et al., '44). The basal diet was fed ad libitum and consisted of whole milk powder<sup>1</sup> supplemented with highly purified iron and copper. Each rabbit received 2 mg of iron and 0.2 mg of copper daily in a little fluid milk. Manganese ( $\text{MnCl}_2$ , c.p.) was fed by mouth daily to those rabbits indicated in the tables. Distilled water was provided in pyrex glass cups. All rabbits were weighed at weekly intervals and carefully observed for symptoms.

The manganese content of the whole milk powder was determined by the method of Gates and Ellis ('47). Samples were taken in duplicate from each of 5 cans. The average value for the duplicates ranged from 0.109 to 0.191  $\mu\text{g}$  per gm. The mean value for the 5 cans was 0.141  $\mu\text{g}$  per gm.

Two experiments were conducted at different times. The first experiment was exploratory, while the second experiment was designed to determine more precisely the quantity of manganese required to prevent the various symptoms. In the first experiment 39 rabbits of mixed sexes and ranging in age from 21 to 24 days of age were divided into 4 groups of 12, 9, 9, and 9 rabbits each. In addition to the above basal diet the groups were fed, respectively, 0, 1, 2, and 4 mg of manganese per rabbit per day. Among those rabbits fed the basal diet alone, 3 died at the respective ages of 10, 12, and 15 weeks. One rabbit in the 4 mg manganese-supplemented group died at 10 weeks of age. The remaining rabbits, with the exception of 4 females fed a 1-mg supplement of manganese and 4 females fed a 4-mg supplement of manganese, were sacrificed at 19 weeks of age. The 8 female rabbits were

<sup>1</sup> Borden's Klim.

continued on the experiment to observe the effects of manganese deficiency on reproduction.

In the second experiment 45 rabbits were distributed at random among 5 equal groups and fed the basal diet supplemented, respectively, with 0, 0.3, 0.6, 1, and 8 mg of manganese per rabbit per day. These rabbits were sacrificed at 20 weeks of age with the exception of 11 that died earlier in the experiment. These 11 rabbits were distributed at random among the groups receiving treatment. These deaths occurred for the most part in the late stages of the experiment (see discussion).

The deficiency symptoms observed in previous studies (Smith et al., '44; Ellis, Smith and Gates, '47) were used as criteria to determine the adequacy of the various levels of manganese fed, namely, gross crookedness of the front leg bones, subnormal growth, length, density and weight of the bones, manganese concentration of the bones and livers, "alkaline" phosphatase activity of the bones and arginase activity of the livers.

The chemical methods used to determine manganese, "alkaline" phosphatase activity and arginase activity are described in detail by Ellis et al. ('47).

The data obtained were reduced by means of the analysis of variance and the *t* test used to determine whether particular differences were significant. Odds greater than 19:1 were accepted as statistically significant while odds greater than 99:1 were considered highly significant. The data presented in this paper are in all cases mean values with their standard errors.

## RESULTS

In both experiments most of the rabbits fed the basal manganese-low diet developed crooked front legs. In the first experiment 7 out of 12 and in the second experiment 7 out of 9 developed crooked front legs that were evident in the intact animals and confirmed by the observation of a bending of the radii and ulnae in the dissected bones. No animal

which received 0.3 mg of manganese per day or more showed any sign of crooked leg bones. Microscopic examination of the humeri bones from those rabbits fed the basal diet showed evidence of depressed bone formation previously described whereas the bones from rabbits fed 0.3 mg of manganese or more were to all appearances normal.

Table 1 summarizes the observations on the length, density and fresh weight of the humeri bones of the various treat-

TABLE 1  
*Summary of the observations on the bones of rabbits  
fed various levels of manganese.*

MN FED	ANIMALS	AV. LENGTH OF HUMERI	AV. DENSITY OF HUMERI	AV. FRESH WT. OF HUMERI
mg/day		cm	gm/ml	gm
First experiment				
0	11	$4.87 \pm 0.101$	$1.329 \pm 0.0194$	$1.988 \pm 0.1479$
1	5	$5.36 \pm 0.163$	$1.366 \pm 0.0133$	$2.191 \pm 0.1878$
2	9	$5.53 \pm 0.101$	$1.399 \pm 0.0119$	$2.416 \pm 0.0881$
4	4	$5.21 \pm 0.121$	$1.361 \pm 0.0507$	$2.093 \pm 0.2839$
Second experiment				
0	7	$4.81 \pm 0.123$	$1.331 \pm 0.0277$	$2.199 \pm 0.1370$
0.3	7	$5.27 \pm 0.097$	$1.385 \pm 0.0146$	$2.437 \pm 0.1013$
0.6	7	$5.31 \pm 0.152$	$1.374 \pm 0.0164$	$2.232 \pm 0.0834$
1	7	$5.56 \pm 0.110$	$1.375 \pm 0.0094$	$2.579 \pm 0.0775$
8	6	$5.55 \pm 0.026$	$1.370 \pm 0.0159$	$2.367 \pm 0.0507$

Analysis of variance showed a highly significant or significant (fresh weight of humeri) difference among the treatment groups in each experiment.

ment groups. In both experiments the length, density and fresh weight of the humeri of the rabbits fed the basal diet were significantly less than the respective values for rabbits fed additional manganese. No significant improvement in bone length, density or weight was obtained by feeding more than 0.3 mg of manganese per day.

The manganese concentration and the "alkaline" phosphatase activity of the ulna bones are given in table 2. The manganese content per unit of dry, fat-free bone was very low in those rabbits fed the basal diet alone as compared with

those rabbits fed additional manganese. The manganese concentration continued to increase with increasing manganese intake within each experiment. It will be noted that the manganese concentration of the bones of the rabbits fed 8 mg of manganese in the second experiment is less than in those rabbits fed 4 mg of manganese in the first experiment. Since these experiments were conducted at different times and with

TABLE 2

*The manganese concentration and alkaline phosphatase activity of the bones of rabbits fed various levels of manganese.*

MN FED	NO. OF ANIMALS	AV. MN CONTENT <sup>1</sup> OF FEMUR	NO. OF ANIMALS	AV. "ALKALINE" PHOSPHATASE ACTIVITY OF ULNAE
mg/day		μg/gm		Units/gm. <sup>2</sup>
First experiment				
				(ends of bones) <sup>2</sup>
0	4	0.60 ± 0.080	6	9.3 ± 1.49
1	4	3.07 ± 0.319	4	32.5 ± 2.62
2	9	5.68 ± 0.668	9	27.8 ± 3.49
4	3	12.91 ± 0.322	3	22.9 ± 2.95
Second experiment				
				(whole bones)
0	7	0.44 ± 0.052	6	5.4 ± 0.70
0.3	7	2.26 ± 0.185	7	10.4 ± 1.59
0.6	7	2.48 ± 0.270	7	9.3 ± 0.70
1	7	2.76 ± 0.170	7	9.2 ± 1.18
8	5	6.71 ± 1.350	6	10.5 ± 1.12

Analysis of variance showed a highly significant or significant (phosphatase activity in second experiment) difference among the treatment groups in each experiment.

<sup>1</sup> Dry, fat-free bone.

<sup>2</sup> Fresh bone.

<sup>3</sup> The 2 end quarters.

different groups of rabbits, one may question whether this is a real difference. The "alkaline" phosphatase activity per unit weight of fresh bone was significantly less in the rabbits fed the basal diet alone as compared to those animals fed additional manganese. No further significant increase in phosphatase activity was observed by feeding more than 0.3 mg of manganese.

Liver samples from the rabbits of the second experiment were taken for arginase activity determinations. The livers from the rabbits fed the basal diet had a significantly lower activity than the groups fed additional manganese. The arginase activity levels of the 0, 0.3, 0.6, 1 and 8 mg groups were, respectively:  $15.6 \pm 1.42$ ;  $24.7 \pm 3.05$ ;  $29.4 \pm 4.05$ ;  $24.6 \pm 3.97$ , and  $31.1 \pm 3.42$  units per gm of fresh liver. At first observation the activity levels of the groups fed additional manganese would appear to be significantly different, but this was not true. The large standard errors indicate the large variability encountered.

TABLE 3  
*Manganese content of the livers of rabbits  
fed various levels of manganese.*

MN FED	NO. OF ANIMALS	AV. MN CONTENT OF LIVERS
<i>mg/day</i>		<i>μg/gm of dry tissue</i>
0	10	$0.9 \pm 0.41$
0.3	8	$6.8 \pm 1.37$
0.6	7	$9.8 \pm 1.68$
1	10	$16.2 \pm 3.05$
2	9	$20.5 \pm 1.74$
4	9	$22.7 \pm 2.61$
8	4	$15.4 \pm 1.45$

The concentration of manganese in the livers of the various treatment groups is summarized in table 3. The low concentration of manganese in the livers of the rabbits fed the basal diet alone is marked. The manganese concentration of the livers increased significantly with increased intakes of manganese up to a level of 1 mg of manganese per day after which the concentration leveled off. The concentration of manganese in the liver would thus appear to reach a maximum at much lower intakes of manganese than do the bones.

The data (table 4) relative to the manganese requirement for maximum growth are not adequate enough to warrant a conclusive statement. It will be noted that in the first ex-

periment where manganese levels of 0, 1, 2 and 4 mg are directly compared, maximum growth was obtained in rabbits fed 4 mg of manganese per day. In the second experiment where manganese levels of 0, 0.3, 0.6, 1 and 8 mg were directly compared, maximum growth occurred in those rabbits fed 1 mg of manganese. Since experiments 1 and 2 were conducted at different times and with different rabbits they cannot logically be directly combined. This is supported by the observation that the rabbits fed 1 mg of manganese in the

TABLE 4  
*Growth of rabbits fed various levels of manganese.*

MN FED	NO. OF ANIMALS	AV. INITIAL WEIGHT	AV. FINAL WEIGHT
mg/day		gm	gm
First experiment			
0	9	288	1140 $\pm$ 52
1	9	285	1130 $\pm$ 107
2	9	288	1240 $\pm$ 82
4	8	282	1390 $\pm$ 63
Second experiment			
0	7	274	990 $\pm$ 90
0.3	7	269	1290 $\pm$ 91
0.6	7	290	1194 $\pm$ 47
1	7	276	1350 $\pm$ 39
8	7	284	1120 $\pm$ 93

Analysis of variance showed a highly significant difference among the treatment groups in each experiment.

first experiment gained significantly less weight than those fed 1 mg of manganese in the second experiment. These limited observations indicate that the manganese requirement for maximum growth is higher than the requirement for normal bone development.

The fact that the rabbits fed 8 mg of manganese gained less weight than the rabbits fed 1 mg of manganese in the second experiment may indicate that 8 mg of manganese per day is on the borderline of toxicity for



The manganese concentration of the blood serum was determined on pooled blood samples of the rabbits in the first experiment by the periodate method. In the second experiment the micromethod of Gates and Ellis ('47) permitted manganese to be determined on the blood serum of individual rabbits. Interpretation of the results is difficult because of the large variability observed and the details are not presented here but are being reserved for more study. In both experiments, however, the rabbits fed the basal diet alone, appeared to have significantly lower concentrations of manganese than the rabbits fed additional manganese. Whether or not a difference existed among rabbits fed varying levels of manganese is not certain.

#### DISCUSSION

The level of manganese taken as being adequate depends upon the criteria used. Insofar as crookedness, length, density, fresh weight, microscopic structure and "alkaline" phosphatase activity of the bones and arginase activity of the livers are concerned, 0.3 mg of manganese per rabbit per day appears to be adequate. The manganese concentration of the livers increased significantly up to an intake of 1 mg of manganese per day while the manganese concentration of the bones increased up to the highest level (8 mg) of manganese fed. The minimum manganese intake necessary for maximum growth requires more study, but it appears to be higher than the amount required for normal bone development.

The relatively high mortality of the rabbits, particularly in the second experiment, is worthy of further comment. This rate of mortality may have been influenced by a cirrhosis of the liver which we have consistently observed in rabbits fed a diet of milk or milk products. This cirrhosis was usually of a mild nature characterized by livers which were mottled and lighter in color than normal. In the extreme form the livers were a very light tan color, fibrous and fatty and apparently similar to the cirrhosis in rabbits described by Rich and Hamilton ('40). The incidence of cirrhosis was not cor-

related with the level of manganese intake. Studies designed to determine the cause of the cirrhosis are in progress.

### SUMMARY

The manganese requirement of rabbits has been studied by feeding a basal diet low in manganese supplemented with various levels of manganese. Insofar as bone development is concerned, it appears that 0.3 mg of manganese per rabbit per day is sufficient to meet normal requirements. The minimum manganese intake necessary for maximum growth requires more study, but it appears to be higher than the amount required for normal bone development.

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# SUPPLEMENTAL RELATIONSHIP BETWEEN PORK PROTEIN AND EGG PROTEIN

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The supplemental relationship of the protein in one food product to that in another is of great practical importance in human nutrition. In 1945 we reported that pork protein had a marked supplemental relationship to bread protein, and the question then arose as to the possibility of a similar relationship between pork protein and egg protein. Published data indicate a higher nutritive value for the protein in eggs than for that in pork. Mitchell, Beadles and Kruger ('27) reported a biological value of 79 for pork tenderloin, and Mitchell ('27) found a value of 74 for pork ham. Mitchell and Carman ('26) found a biological value of 94 for dried whole eggs, and 83 for egg albumin. Sumner ('38) found values of 97 for commercially-dried eggs fed to young rats and 85 for such eggs fed to year-old rats when the diet contained 8% of protein. When the diet contained 5% of protein, the biological value of these eggs fed to mature rats was 94. Sumner and Murlin ('38) reported a value of 95 for the protein in dried eggs fed to year-old rats when the diet contained 5% of protein, and a value of 65 when the eggs were consumed by human subjects on diets containing 3 to 4% of protein. Hoagland and Snider ('46) found the average growth-promoting value of the protein in dehydrated pork to be 3.35 gm gain per gm of protein consumed in tests with young rats, as com-

pared with a gain of 4.28 gm for the protein in spray-dried eggs.

The reason for the higher nutritive value of the protein in whole eggs than in pork is, presumably, a better balance between the essential amino acids in eggs and the requirements of the rat. A comparison of available data for the amino acid content of eggs with those for pork, as reported by Beach, Munks and Robinson ('43), Mitchell and Block ('46), and Lyman et al. ('46), indicates that the protein in whole eggs and in egg albumin contains notably more cystine and methionine than the protein in pork. It seemed probable, therefore, that there would be a supplemental relationship between the protein in eggs and that in pork. The purposes of the experiments herein reported were (1) to determine the growth-promoting values of the protein in mixtures of pork and eggs as compared with the values of each product alone, and (2) to determine the effects of the addition of cystine or methionine on the growth-promoting value of the pork protein.

#### EXPERIMENTAL

##### *Products tested*<sup>1</sup>

Three lots of cured and 1 lot of fresh hams were used in feeding tests with rats. Cured ham lot no. 4320 had been cured by the dry-cure method, and cured ham lots nos. 4462 and 4464 were mixtures of hams that had been cured by the dry-cure, artery-cure, and brine-cure methods. The lean meat from each lot of hams was ground and then baked in glass baking dishes in an oven at 325° to 350°F. until the internal temperature of the meat was 155°F. The cooked ham was ground, spread on trays, and dehydrated at 155°F. to a moisture content of 10% or less. The dried meat was thoroughly extracted with ethyl ether, ground fine, and stored in tightly covered jars at approximately 25°F.

<sup>1</sup> Acknowledgment is made to R. L. Hiner for curing and dehydrating the hams and for dehydrating the hard-boiled eggs and to Edna H. Steely for assistance in the statistical analyses. Our thanks are extended to T. C. Byerly and E. H. McNally for supplying fresh eggs and commercially dehydrated eggs.

Two lots of commercial spray-dried eggs (uncooked), 2 lots of dried hard-boiled eggs, and 1 lot of dried whites from hard-boiled eggs were tested. The dried hard-boiled eggs were prepared as follows: The eggs were boiled 10 minutes, chilled, and the contents were ground through a meat grinder, spread on trays, and dried in the same manner as the hams. The dried, cooked whole eggs were thoroughly extracted with ethyl ether, but the spray-dried eggs were not extracted. The dried eggs and egg white were ground fine and stored under the same conditions as the dried ham.

### *Diets fed*

Dehydrated ham, eggs, and egg white were incorporated in diets in such proportions that each product or mixture supplied 1.6% of nitrogen. The following quantities of B vitamins were added to 100 gm of diet in addition to any vitamins present in the ham or eggs: thiamine hydrochloride, 0.3 mg; riboflavin, 0.3 mg; pyridoxine hydrochloride, 0.6 mg; calcium pantothenate, 1.5 mg; and choline chloride, 20 mg. To 1 diet containing spray-dried eggs was added 5  $\mu$ g of biotin. Cystine and methionine were added to certain diets as indicated in the tables.

The fat-soluble vitamins were added in solution in lard so that 1 gm of the diet would contain 5 I.U. of vitamin A, 1 I.U. of vitamin D, and 20  $\mu$ g of alpha-tocopherol. Sufficient kettle-rendered lard was added to make 10% of fat in the diet. Salt mixture amounting to 2%, sufficient sodium chloride to equalize the salt content of all diets in a series including cured ham, and dextrin to make 100% completed the diet. In experiment no. 4 (table 3) which did not include cured ham, 4% of salt mixture was added to the diet.

### *Feeding tests*

Each diet was fed to 8 male albino rats for 30 days. The animals weighed approximately 40 gm each and did not exceed 25 days of age at the beginning of the tests. Rats from

different litters were distributed among the several groups in a series. Each rat was kept in an individual cage which was provided with a raised screen bottom, a self-feeder, and a drinking vessel. The bottom of the cage was covered with a sheet of blotting paper. The rats were weighed twice weekly and the feeders were refilled about every third or fourth day. A record was kept of the feed consumed. The temperature of the rat laboratory was approximately 75°F.

### RESULTS

The results of experiment no. 1, table 1, indicate that the protein in 1 lot of spray-dried eggs was superior in growth-promoting value to the protein in dry-cured ham. This is true not only on the basis of gain in weight but more certainly on the basis of gain in weight per gram of nitrogen consumed, the criterion primarily employed in this present study for evaluating the diets. However, when the diet contained equal parts of ham and egg protein, the growth-promoting value was practically the same as for eggs alone, and definitely higher than the computed value. When the diet contained 2 parts of ham and 1 part of egg protein, the growth-promoting value was somewhat higher than the computed value, but lower than the value for eggs alone. The results of this experiment indicate a moderate supplemental relationship between the protein in dry-cure ham and that in cooked, spray-dried eggs.

Statistical tests by means of Fisher's "t" values for the significance of differences between the means of the gain in weight per gram of nitrogen consumed showed the following probability values:

Diet 1 with diets 2, 3, and 4	— very highly significant ( $< .001$ )
Diet 4 with diets 2 and 3	— highly significant ( $< .01$ )
Diet 2 with diet 3	— not significant ( $> .6$ )

In experiment no. 2, further tests were made on ham alone, eggs alone and combinations of ham and eggs, together with supplements of biotin, methionine and cystine added to cer-

TABLE 1

*Supplemental relationship between the protein in cured ham and that in spray-dried eggs in 30-day tests with groups of 8 male rats.*

SOURCE OF NITROGEN IN DIET	DIET NO.	GAIN IN WEIGHT		FEED CON- SUMED	NITRO- GEN IN DIET	GAIN IN WEIGHT PER GRAM OF NITROGEN CONSUMED	
		Ac- tual	Com- puted <sup>1</sup>			Ac- tual	Com- puted <sup>1</sup>
		gm	gm	gm	%	gm	gm
Experiment no. 1 (dry-cured hams)							
Dry-cure ham no. 4320	1	105	.	320	1.60	20.48	
Dried eggs no. 4343	2	111		285	1.60	24.26	
Dry-cure ham no. 4320, 0.8% N + dried eggs no. 4343, 0.8% N	3	123	108	318	1.60	24.04	22.37
Dry-cure ham no. 4320, 1.067% N + dried eggs no. 4343, 0.533% N	4	117	107	324	1.60	22.62	21.74
Experiment no. 2 (hams cured by several methods)							
Cured ham no. 4462	1	101		316	1.60	19.93	
Cured ham no. 4462 + 0.2% methionine	2	129		333	1.62	23.66	
Dried eggs no. 4463	3	110		292	1.60	23.58	
Dried eggs no. 4463 + 5 µg biotin in 100 gm diet	4	111	.	291	1.60	23.76	
Dried eggs no. 4463 + 0.20% methionine	5	115	.	296	1.62	24.00	.
Cured ham no. 4462, 0.8% N + dried eggs no. 4463, 0.8% N	6	119	106	324	1.60	22.88	21.76
Cured ham no. 4462, 0.8% N + dried eggs no. 4463, 0.8% N + 0.2% cystine	7	130	106	326	1.62	24.51	21.76
Cured ham no. 4462, 1.067% N + dried eggs no. 4463, 0.533% N	8	116	104	333	1.60	21.53	21.06
Cured ham no. 4462, 1.067% N + dried eggs no. 4463, 0.533% N + 0.2% cystine	9	134	104	339	1.62	24.27	21.06

<sup>1</sup> The computed gain of rats fed a mixture of ham and eggs is the weighted average of gains made by rats fed the individual products.



tain ones of these diets. Statistical tests similar to those given for experiment 1 are summarized as follows:

Diet 1 with diets 2, 3, 4, 5, 6, 7, and 9—very highly significant ( $< .001$ )

Diet 8 with diets 2, 3, 5, 7, and 9—highly significant ( $< .01$  or  $.02$ )

Diet 8 with diet 4—significant ( $< .05$ )

Diet 6 with diet 7—highly significant ( $< .02$ )

Diet 6 with diet 5—significant ( $< .05$ )

Differences between all other diets— not significant.

The data given in table 1 indicate that the protein in a second lot of spray-dried (diet 3) eggs was also higher in growth-promoting value than the protein in a composite lot of cured hams (diet 1). However, when this lot of ham was supplemented with methionine, the nutritive value of the protein was increased to an approximate equality with the protein in eggs. It is noteworthy that the actual gain in weight of the rats on the diet containing ham and methionine was considerably larger than for the rats on the diet containing eggs alone. The addition of biotin to the egg diet was without material effect on growth, and the addition of methionine was followed by an insignificant increase.

When the diet contained equal parts of ham and egg protein (no. 6), the growth-promoting value was moderately higher than the computed value, but lower, although not significantly, than the value for eggs alone. When this diet was supplemented with cystine, the nutritive value was increased above that for eggs but not enough to show statistical significance.

The growth-promoting value of diet 8 containing 2 parts of ham and 1 part of egg protein was only slightly higher than the computed value, but the addition of cystine definitely increased the nutritive value of the protein above that for eggs.

The results of experiment no. 2 indicate only a moderate supplemental relationship between the protein in cured ham and that in uncooked, spray-dried eggs. The supplemental value of methionine for pork protein, and of cystine for mixtures of pork and egg protein, is clearly apparent.

In table 2 are shown the results of experiment no. 3 with cured ham, dried hard-boiled eggs, and with mixtures of these foods. A summary of the statistical comparisons shows the following:

Diet 1 with diets 2, 3, 4, 5, 6, 7, and 8—very highly significant ( $< .001$ )

Diet 6 with diet 7—highly significant ( $< .01$ )

Diet 6 with diets 2 and 5—significant ( $< .05$ )

Diet 8 with diets 2, 3, 5, and 7—highly significant ( $< .01$ )

Differences between all other diets—not significant.

The growth-promoting value of the protein in eggs was definitely higher than that for ham, but when ham was supplemented with methionine, the nutritive value of the protein was

TABLE 2

*Supplemental relationship between the protein in cured ham and that in dried, hard-boiled eggs in 30-day tests with groups of 8 male rats (experiment no. 3).*

SOURCE OF NITROGEN IN DIET	DIET NO.	GAIN IN WEIGHT		FEED CON- SUMED	NITRO- GEN IN DIET	GAIN IN WEIGHT PER GRAM OF NITROGEN CONSUMED	
		Ac- tual	Com- puted			Ac- tual	Com- puted
Cured ham no. 4464	1	gm 108	gm .	gm 339	% 1.60	gm 19.77	
Cured ham no. 4464 + 0.2% methionine	2	125		327	1.62	23.44	
Dried eggs no. 4466	3	129		344	1.60	23.40	
Dried eggs no. 4468 + 0.2% methionine	4	135		336	1.62	24.86	
Cured ham no. 4464, 0.8% N + dried eggs no. 4466, 0.8% N	5	139	119	373	1.60	23.27	21.59
Cured ham no. 4464, 0.8% N + dried eggs no. 4466, 0.8% N + 0.2% methionine	6	142	119	354	1.62	24.72	21.59
Cured ham no. 4464, 1.067% N + dried eggs no. 4466, 0.533% N	7	139	115	375	1.60	23.09	20.98
Cured ham no. 4464, 1.067% N + dried eggs no. 4466, 0.533% N + 0.2% methionine	8	140	115	342	1.62	25.24	20.98

practically the same as that of eggs. When eggs were supplemented with methionine (diet 4), there was an apparent slight increase in growth-promoting value. A mixture of equal parts of ham and egg protein had practically the same growth-promoting value as eggs alone, and a higher value than the computed one. When this mixture was supplemented with methionine, the nutritive value was materially increased. Diet 7 containing 2 parts of cured ham protein and 1 part of cooked egg protein induced nearly as efficient growth as eggs alone, and much better growth than the computed value. When this diet was supplemented with methionine (diet 8), the growth-promoting value was significantly increased.

The results of experiment no. 3 indicate a definite supplemental relationship between the protein in hard-boiled eggs and that in cured ham, but the growth-promoting value was further improved by the addition of methionine. The nutritive value of the protein in cured ham was increased to that of cooked eggs by the addition of methionine.

In table 3 are shown the results of experiment no. 4 concerning the supplemental relationship between the protein in dried, hard-boiled egg white and that in fresh ham. The statistical comparisons gave the following:

Diet 1 with diets 2, 3, 4, 5, and 6 — very highly significant ( $< .001$ )

Diet 6 with diets 3 and 5 — very highly significant ( $< .001$ )

Diet 6 with diet 4 — highly significant ( $< .01$ )

Differences between all other diets — not significant.

The growth-promoting value of egg white protein in diet 3 was materially higher than that of fresh ham, but when the latter was supplemented with cystine, the nutritive value was increased above that for egg white alone although not enough to be significant (diet 2 versus diet 3). A mixture of equal parts of ham and egg white protein in diet 4 did not give a significant increase in nutritive value over that of egg white alone. Similar results were obtained with a mixture of 2 parts of ham and 1 part of egg white protein. However, when the latter mixture was supplemented with cystine (diet 6), the growth-promoting value was definitely increased.

None of the rats in experiment no. 4 with cooked egg white, or in the previous experiments with either raw or cooked eggs, showed any symptoms of biotin deficiency. At the end of the 30-day test with the rats on the diet containing 10% of egg white protein, 2 rats were continued on the same diet for 30 days longer during which period each rat was fed 6  $\mu$ g of biotin weekly. No increase in rate of growth was observed.

TABLE 3

*Supplemental relationship between the protein in fresh ham and that in dried, hard-boiled egg white in 30-day tests with groups of 8 male rats (experiment no. 4).*

SOURCE OF NITROGEN IN DIET	DIET NO.	GAIN IN WEIGHT		FEED CON- SUMED	NITRO- GEN IN DIET	GAIN IN WEIGHT PER GRAM OF NITROGEN CONSUMED	
		Ac- tual	Com- puted			Ac- tual	Com- puted
Fresh ham no. 4471	1	gm 111	gm	gm 332	% 1.60	gm 20.83	
Fresh ham no. 4471 + 0.4% cystine	2	141		352	1.65	24.26	
Dried egg white no. 4470	3	126		343	1.60	23.00	
Fresh ham no. 4471, 0.8% N + dried egg white no. 4470, 0.8% N	4	128	119	342	1.60	23.36	21.97
Fresh ham no. 4471, 1.067% N + dried egg white no. 4470, 0.533% N	5	126		338	1.60	23.17	21.56
Fresh ham no. 4471, 1.067% N + dried egg white no. 4470, 0.533% N + 0.2% cystine	6	144		347	1.62	25.55	21.56

The results of experiment no. 4 indicate a definite supplemental relationship between the protein in fresh ham and that in cooked egg white, but the growth-promoting value of a mixture was increased above that for egg white alone by the addition of cystine.

## SUMMARY

The supplemental relationship between the protein in pork and that in eggs, and the effectiveness of cystine and meth-

ionine in correcting the deficiency in pork, were determined by experiments with young male albino rats with the following results:

The protein in both fresh and cured hams contained insufficient cystine and methionine for optimum growth in rats when the diets contained 10% of protein; but when pork protein was supplemented with these amino acids, the growth-promoting value was equal to that of protein in eggs.

There was found to be a moderate supplemental relationship between the protein in ham and that in both whole eggs and egg whites, but this relationship was materially improved by the addition of cystine or methionine. Mixtures of equal parts of pork and whole egg protein had approximately the same growth-promoting value as egg protein alone, and similar results were obtained with mixtures of equal parts of pork and egg white protein.

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# EFFECT OF ENVIRONMENT ON GROWTH AND FEED AND WATER CONSUMPTION OF CHICKENS

## I. THE EFFECT OF TEMPERATURE OF ENVIRONMENT DURING THE FIRST NINE DAYS AFTER HATCH

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### FIVE FIGURES

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### INTRODUCTION

In recent studies with the respiration calorimeter, Barott and Pringle ('46) noted that temperature of environment had an important effect on the energy and gaseous metabolism of chickens. Baby chicks at an environmental temperature of 70°F. eliminated 2.35 times as much heat as at 95°F., 5-week-old chickens 1.85 times as much at 45°F. as at 95°F., etc.

Homoiotherms consume more food at low temperatures than at high ones. This must be true because at the lower temperatures more food must be metabolized to regulate body temperature. However, the capacity of an animal to consume and metabolize foodstuffs is limited; therefore, one would expect that there would be a minimum temperature below which an animal would perish because it could not metabolize enough food for maintenance. At very high temperatures an animal does not grow well, probably because of insufficient food consumption, and above a certain point perishes because it is not

able to cool itself sufficiently to maintain body temperature. Between the minimum and maximum for existence there must be a temperature at which the efficiency, i.e., the growth increment divided by the food intake, is a maximum.

Because there are few data on the effect of temperature and other physical factors of environment on growth and feed and water consumption of chickens, an investigation of this subject was undertaken at the Agricultural Research Center. Since such an investigation must be extensive to cover the life cycle of the chicken and study the effect of each factor (temperature, humidity, light, etc.) separately for all ages, it was decided to publish the results as they were obtained. The temperature factor is undoubtedly the predominant influence; therefore, its effect was studied first and this paper discusses the results obtained with various temperatures, chickens from time of hatch to 9 days of age being used.

A search of the literature reveals a dearth of information on growth and feed consumption for the first few days after hatch, and as far as could be determined no values have been published on the day-by-day efficiency of feed utilization during the first week.

The economic importance of determining the optimum conditions for brooding can be easily understood from the following: In 1945 there were hatched in the United States alone 1,600,000,000 chicks with a monetary value of approximately \$180,000,000. A study by the Ohio Poultry Improvement Association ('43-'44), of 8,317 groups totaling 2,400,000 chickens showed a mortality of 4% during the first 3 weeks after hatch. These were selected groups from large commercial hatcheries. Over the country as a whole, the loss would undoubtedly run considerably higher. If, by increased knowledge of proper environmental conditions for brooding, even one-fourth of this loss could be prevented, and investigation indicates that it could be, a saving of approximately \$2,000,000 per annum would be affected.

## APPARATUS

For the proper conduct of such an investigation, the chickens under observation must be housed in such a manner that all the physical factors relative to environment are under accurate control at all times and that any 1 factor may be varied at will while the other factors remain unchanged.

A room of sufficient size to accommodate the necessary equipment was, therefore, selected. This room is equipped with air-conditioning apparatus to control both the temperature and humidity. A description of the control apparatus for this room has been published by Barott ('39).



Fig.1 Exterior of the 3 houses used in the experiments.

Three small houses (fig. 1) were built within this room. Each is 5 feet by 6 feet by 7 feet high. The walls, floor and ceiling are constructed of compressed cork board 2 inches thick, covered on either side with  $\frac{1}{4}$ -inch wall board which is painted white. Each house is fitted with apparatus for the accurate control of all the physical factors to be investigated.

Figure 2 shows a view of the interior of one of these houses. For temperature control, an electric heater coil (fig. 2a) is supported from the frame of a fan (fig. 2b) in such a manner that the air from the fan passes over the turns of the coil. The power supply to this heater is controlled by a bi-metallic



thermostat (fig. 2c) which may be set for any temperature between  $0^{\circ}$  and  $200^{\circ}\text{F}$ . and has a sensitivity of  $\pm 0.35^{\circ}\text{F}$ . The fan is in motion at all times whether the heater is on or off, in order to keep the air in the house well stirred so that there will be little temperature or humidity gradient.

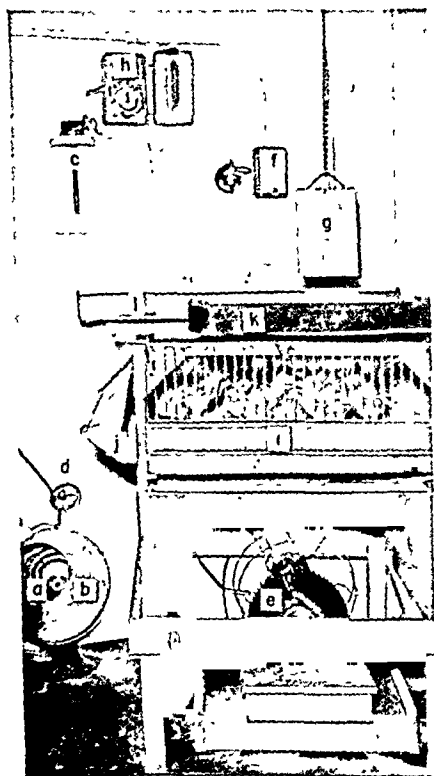


Fig. 2 Interior of one of the houses with controlled environment.

For ventilation purposes there are 2 vents in the ceiling and a vent with a damper in the rear wall (fig. 2d). By adjusting these apertures as much fresh air as desired may be admitted.

The humidification of the air is accomplished as follows: A light copper wheel (fig. 2e), 9 inches in diameter with 64 vanes, each 3 inches by 3 inches, is mounted on ball bearings in such a manner that as the wheel rotates the vanes dip into

a pan of water which is covered except for the opening for the wheel. This wheel is made to rotate by the air from a fan which is placed at the rear of the wheel. As the dampened vanes of the rotating wheel come within the air stream from the fan, sufficient water is vaporized and carried away in the air stream to bring the humidity inside the house to any predetermined level. Humidity control is by the humidistat (fig. 2f), which controls the power supply to the fan. When the humidity reaches the desired level the humidistat opens the fan motor circuit, causing the fan to stop and, therefore, also the wheel for humidification. As soon as the humidity drops slightly, the humidistat again starts the fan and thus the paddle wheel. By this means the humidity is always kept at the desired level.

A continuous record of temperature and humidity was kept on a chart of a Friez Recorder (fig. 2g) suspended directly over each cage. The recording apparatus was calibrated before the experiment started. The temperature graph was checked daily by a calibrated mercury thermometer and the humidity by an aspirating psychrometer.

A white fluorescent lamp, 4 feet long, of 40 watt capacity, attached to the ceiling directly over the cages, was used in each of the houses. Any type or color of light can be used by changing the bulb or, if necessary, the fixture. The light periods (of which there were 3 or 4) were controlled by a time clock (fig. 2h). The total duration of these periods was 12 hours out of each 24 hours.

#### PROCEDURE

The chickens used were from selected flocks of Rhode Island Reds. The breeding birds were maintained and the eggs hatched at the Agricultural Research Center. The chicks were removed from the hatcher when the average age was about 12 hours. They were not sexed but it developed that they were fairly evenly divided between the 2 sexes. A total of 300 to 400 chicks were provided for each test from which 90 of as nearly identical weight as possible were selected. The

average weight of all the chicks used was 40.5 gm, with a mean deviation in weight for the lot of approximately 1 gm. After selection, the chicks were leg banded and divided into 3 lots of 30 each. One group of 30 chicks was placed in each of the 3 houses in which the environmental conditions for the experiment had already been established. The chicks were brooded without a hover; consequently, they were at all times in an atmosphere of uniform temperature and humidity.

The following routine was observed at the same time each day. The chicks were weighed individually and as a unit and placed in the brooder. The water troughs (fig. 2i) were scrubbed and fresh water poured into them. Enough feed was added to the feed troughs (fig. 2j) to bring the feed in them up to the desired level. The troughs were weighed after these additions and the observed weights recorded as the initial weights of a 24-hour period. At the end of 24 hours, the chicks were removed from the brooder and weighed. The difference between this weight and the weight at the start of the period gave the gain in weight for that period. The feed and water troughs were also weighed at the end of 24 hours.

The difference between the initial and final weights equalled the amount of feed eaten and water drunk after correction for evaporation of water from the water trough and evaporation or absorption of water vapor from the atmosphere by the feed troughs. Correction factors for this gain or loss of water were obtained by using 2 check troughs (fig. 2k and 2l) identical in shape and size to those used for the chicks. These troughs were provided with the same amount of feed and water as the regular troughs but were placed out of reach of the chicks. The change in weight of these troughs was obtained each day and applied as a correction factor to the amount of water drunk and feed eaten. The diet (with the addition of 1 mg of synthetic riboflavin per kilogram of feed) fed to the chicks throughout the experiments had the following percentage composition: ground yellow corn, 37.575; ground wheat, 20.00; ground oats, 10.00; alfalfa leaf meal, 3.00; soybean meal, 20.00; sardine meal, 5.00; dried skim

milk, 2.00; ground limestone, 1.00; steamed bonemeal, .90; salt (96% NaCl; 4%  $\text{mn.S}_4$ ), .50, and vitamin D powder (2000  $\mu\text{g/gm}$ ), .025.

The first experiment was started May 22, 1944, and 72 experiments have been made with chicks from time of hatch to 9 days of age. As the environment in each small house was controlled independently of the other houses, it was possible to conduct 3 experiments simultaneously. Thirty chicks were placed in each house during an experiment; consequently the work herein reported comprises data involving the use of 2,160 chicks. Mortality was practically nil. Occasionally a chick was caught in the wire floor and injured so that it had to be destroyed, but no deaths were due to infections or disease and none to environment.

When the analysis of the results was started, the question arose as to how to obtain the true gain in weight from day to day. Before hatching, the chick takes the yolk of the egg into the body cavity to be used as food material during the first few days after hatching; thus for several days the system contains decreasing amounts of this substance. When the chick starts to eat, undigested and unassimilated feed accumulates in the digestive tract. This feed and yolk are not properly a part of the chick until they are metabolized and utilized for body building. Therefore, it was decided to eliminate these factors and use the true increase in weight as a basis for analysis of the effect of temperature of environment.

To determine the quantity of extraneous matter, 30 chicks were dissected each day from the first day after hatching to the tenth day. They were dissected after a dark period (consequently after a period of no feeding) so that the crop was invariably empty. All weights used in these experiments were taken after a dark period of 4 hours so that the amount of feed in the system of the chick was comparable to that found by this dissection. The yolk sac and yolk and the contents of gizzard and of the intestinal tract of each chick were carefully isolated and weighed. A mean value for each of these quantities was taken for each day and the total amount was

used as a correction factor to the weight of the chick. In other words, the observed weight, minus foodstuffs within the body, gave the true weight for the purpose of analysis.

### RESULTS AND DISCUSSION

Figure 3 shows the mean weight each day of (1) yolk sac and contained yolk, (2) gizzard contents and (3) intestinal contents. Curve 4 shows the sum of these 3 quantities and it was from this curve that the values were obtained which were subtracted from the observed weight of the chick each day.

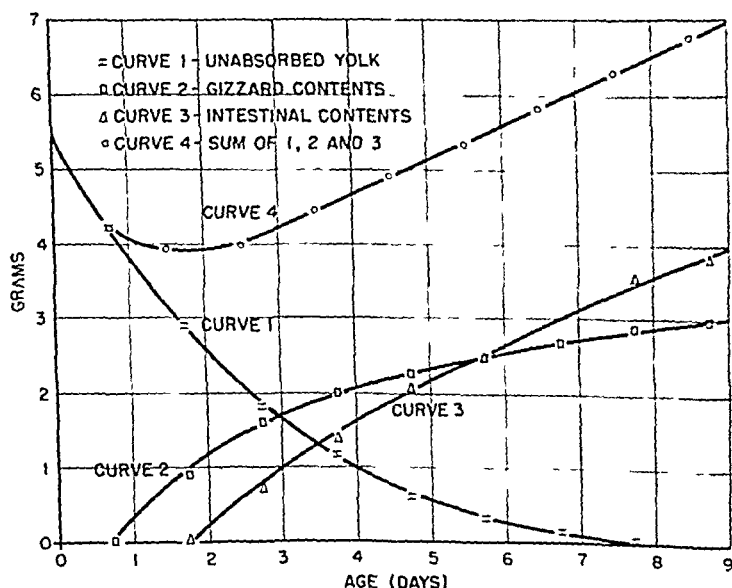


Fig. 3 Correction applied to chick weight for extraneous matter in system.

The difference in these corrected weights gave the true gain in weight from day to day.

Little is known about the optimal environmental temperature for brooding. United States Department of Agriculture Farmers Bulletin No. 1538 states that it is the custom to start brooders at about 95°F., and that in those with small hovers the temperature is reduced 3.5° to 5.0°F. weekly down to 70°F.

The heat is ordinarily cut off after the chick is well feathered. Electrically heated battery brooders are usually operated in a room where the temperature is maintained at from 60° to 70°F. Of course, this does not give much information as to optimal brooding temperature, for the chick can change position from the hover to the cooler environs at will. Whether the chick grows better and is more sturdy when brooded at a uniform temperature or as ordinarily with a hover is one of the questions which will be studied. All that can be said at present is that there was a mortality of less than 1% the first 9 days when the chicks were brooded without a hover, the chicks were well feathered and the growth was satisfactory.

In the experiments herewith reported the temperature ranged from 96° to 85°F. for the first day after hatch and from 92° to 80° for the ninth day after hatch.

The humidity was kept between 60 and 70% in all experiments and the feeding time was approximately 12 hours out of each 24-hour day. The total feeding time was divided into 3 or 4 periods.

The data obtained for each experiment were computed for (1) daily gain in weight in grams and in percentage increase over the original weight, (2) grams of feed consumed per chick per day, (3) grams of water consumed per chick per day.

From comparative analyses of the results obtained in all the experiments which were conducted at any given temperature, the percentage of gain in weight of the chicks for 9 days was obtained. The results for each temperature were plotted and curve 1, figure 4, constructed through the plotted points. The numeral at each point designates the number of experiments used to obtain that point, e.g., a point with the figure 11 designates 11 experiments of 30 chicks each or a mean for 330 chicks. The point is plotted at the temperature which is a mean for that group. The range in temperature for each group can be determined from table 1.

Curve 1, figure 4, shows a very definite variation in growth rate due to temperature. The maximum growth was obtained when the mean temperature was 91°F. and equalled a 59%

increase over the original weight in 9 days. This occurred when the chicks were kept in an environmental temperature of 94°-95°F. the first day and thereafter the temperature was decreased uniformly to 88°F. on the ninth day. The gain in weight was less at other temperatures studied and became

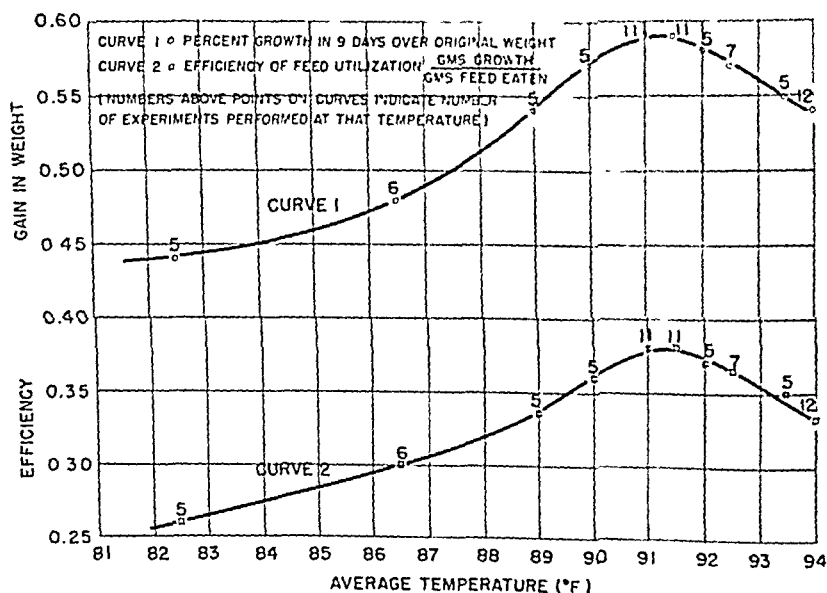


Fig. 4 Effect of temperature of environment on growth and efficiency of feed utilization during first 9 days of age.

TABLE 1

*Number of experiments at temperatures studied.*

NUMBER OF EXPERIMENTS	TEMPERATURE 1ST DAY (°F)	TEMPERATURE 9TH DAY (°F)
12	96	92
5	95	91
7	94	91
5	96	88
11	95	88
11	94	88
5	92	88
5	93	84
6	90	83
5	85	80

less and less the farther the deviation below or above the temperature where maximum growth occurred, until at a temperature of 82.5°F. (initial temperature 85°F., final temperature 80°F.), the increase in weight was only 44%.

Two experiments were performed with a constant temperature throughout the 9 days, 1 at 93°F., and 1 at 91°F. At 93°F. a 54% increase in weight over the 9 days was noted; at 91°F. a 55% increase. Since in each of these experiments the growth in 9 days was less than when the temperature was decreased day by day it was concluded that it was less satisfactory to maintain a constant temperature.

Kleiber and Dougherty ('33) published the results of a well-conducted experiment on the effect of environmental temperature on growth and feed consumption between 5 and 15 days of age. They found that the lower the temperature between 21° and 40°C. (69.8° and 104°F.) the better the growth. This was not true in our experiments with chicks between time of hatch and 9 days of age. However, it might be expected that younger chickens would need a higher temperature for satisfactory growth.

The *ad libitum* feed consumption per chick (a surplus of feed was before the chicks at all times so they could eat as much as they desired) was practically constant in amount between the temperatures of 82° and 94°F. Therefore, curve 2, figure 4, which shows the efficiency of the chick in feed utilization (grams gain in weight divided by the grams of feed eaten), has practically the same form as curve 1. At 91°F. the gain in weight equalled approximately 0.38 gm per gm of feed eaten. As the temperature was lowered the rate of growth became less and less so that at an average temperature of 82°F. the efficiency had dropped to 0.26 gm of growth per gm of feed. The efficiency also became increasingly less as the temperature was raised above 91°F. until at 94°F., it was 0.33 gm of growth per gm of feed eaten.

After the range of temperature was determined within which the growth of the chicks was maximum, results from the 22 experiments (660 chicks) conducted within this range



were analyzed to obtain the values shown by curves 1 to 4, inclusive, in figure 5.

Curve 1, showing the increase in weight each day in grams, seems to be nearly linear for the first 5 days; thereafter the rate appears to accelerate.

Curve 2 showing percentage increase over original weight, is the typical growth curve for chicks. Growth starts slowly for the first days after hatch and accelerates each day until

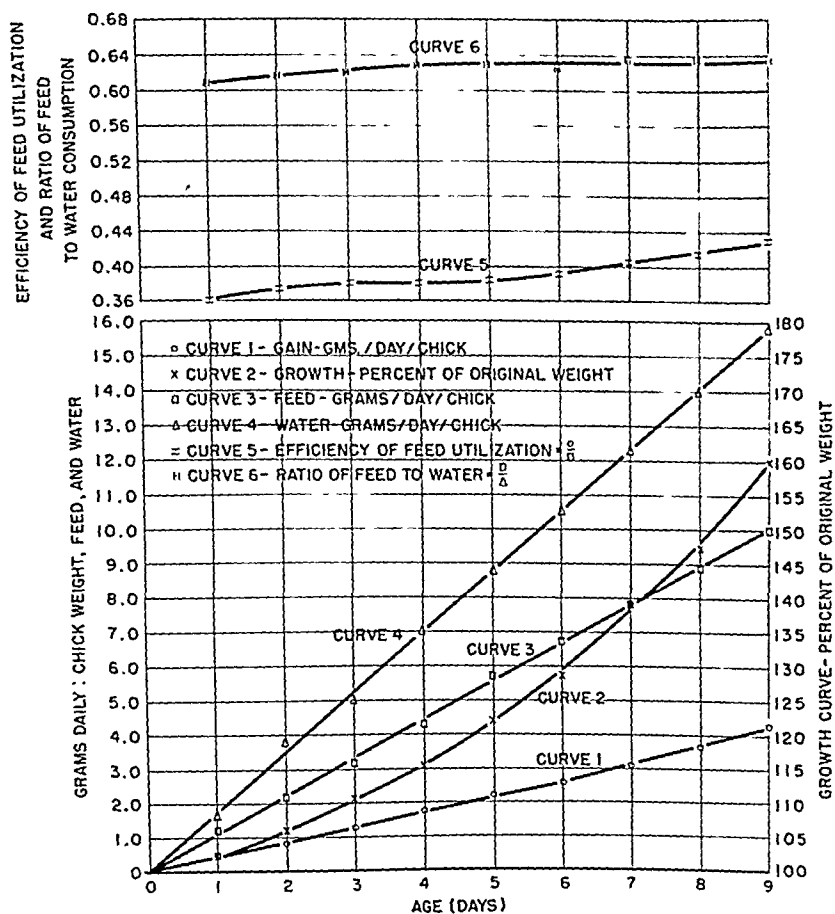


Fig. 5 Growth and feed and water consumption of chickens from hatch to 9 days of age.

the growth between the eighth and ninth days is 6 times greater than that between the start and the first day. The growth between the eighth and ninth days was over 10% of the weight on the eighth day.

Curves 3 and 4, showing feed and water consumption, appear to be lineal, the amount consumed each day being approximately 1.0 gm of feed per chick more than was consumed the previous day and 1.7 gm of water per chick more than the previous day.

From curves 1 and 3 (fig. 5), it was possible to compute the efficiency of the chick in the utilization of foodstuffs. The daily gain in weight in grams (from curve 1) divided by the daily feed consumption (from curve 3) equals the efficiency. Results obtained in this manner are shown in curve 5, drawn through the plotted points. On the first day the efficiency was found to be 0.36. It rose to 0.38 on the third day, remained practically constant at that figure on the fourth and fifth days and rose to 0.43 on the ninth day.

Kleiber and Dougherty ('33) found an efficiency of feed utilization, based on dry weight of feed, of 0.375 at 32°C. (89.6°F.) for chicks between 5 and 15 days of age. In the investigation herein reported with chicks between time of hatch and 9 days of age an efficiency of 0.35 was found at this same temperature. The feed given to these chicks contained 10% of moisture. When the feed was calculated on a dry weight basis, the efficiency of the chicks at 32°C. (89.6°F.) was 0.39.

Hammond et al. ('38) indicated the efficiency of feed utilization as a straight line with a maximum value at hatch and decreasing to zero when growth ceases. This conclusion was based on weekly weights of chickens. The efficiency reported herein is least at hatch and increases gradually for the first 9 days. It will ultimately become stable and then begin to decrease because the efficiency when growth ceases is zero.

Curve 6 (fig. 5), shows the relation between feed consumed and water drunk, i.e., grams of feed consumed per chick per day, divided by the grams of water drunk per chick per day.

This curve starts at 0.61, rises to 0.63 on the fourth day and remains constant at this figure to the ninth day. On the ninth day after hatch, the chick consumed approximately 2.35 gm of feed to increase its weight 1 gm and drank approximately 1.6 gm of water for each gm of feed eaten.

#### SUMMARY

Chicks were housed in controlled environment without a hover for the first 9 days after hatch in order to determine the optimum temperature for growth and efficiency of feed utilization. Thirty chicks were used in each experiment and a total of 72 experiments were performed. Mortality for all experiments was practically nil. Relative humidity was kept between 60 and 70% in all experiments. The chicks were allowed 12 hours ad libitum feeding time, divided into 3 or 4 periods with an equal dark period between each feeding period.

The temperature was varied with different lots of chicks, from a mean temperature of 94°F. over the 9 days (96° for the first day and gradually reduced to 92°F. the ninth day) to a mean temperature of 82.5°F. over the 9 days (85°F. the first day and gradually reduced to 80°F. the ninth day).

Maximum growth and efficiency of feed utilization were noted when the temperature was 94°-95°F. the first day dropping uniformly to 88°F. on the ninth day. The growth under these conditions equalled a 59% increase over the original weight in 9 days. (All weights of chicks were obtained 4 hours after the last feeding period, and therefore there was no feed in the crop.) The mean efficiency of feed utilization over the 9 days was 0.38. Both growth and efficiency became less as the temperature varied from this range: the greater the deviation, the greater the difference, until at 82.5°F. (85° to 80°F.) the increase in weight was only 44% and the efficiency 0.26. At 94°F. (96°-92°F.) the increase in weight was 54% and the efficiency 0.33. The amount of feed consumed increased approximately 1 gm per day per chick. On the ninth day after hatch the chick consumed approximately 2.35 gm of feed for

every gm increase in weight and drank approximately 1.6 gm of water for each gm of feed consumed.

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# RIBOFLAVIN EXCRETIONS AND TEST DOSE RETURNS OF YOUNG WOMEN DURING PERIODS OF POSITIVE AND NEGATIVE NITROGEN BALANCE

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An inverse relationship between the protein intake and the urinary excretion of riboflavin in dogs and rats was demonstrated by the studies of Sarett and associates ('42, '43). They found that a decrease in the protein content of the diet, associated with a negative nitrogen balance produced an increase in the urinary excretion of riboflavin; an increase in the protein content of the diet, associated with a positive nitrogen balance, produced a decrease in the urinary riboflavin excretion. That such a relationship also existed in children was indicated in a study carried out in our laboratory (Oldham et al., '44). When 2 pre-school children were on low-protein intakes during the course of acute upper respiratory infections, their urinary riboflavin excretions increased; when they returned to normal protein intakes, their riboflavin excretions decreased despite the fact that their riboflavin intakes had been maintained at a constant level. Both the reduced protein intakes and the elevations in body temperature which accompany infections usually tend to produce negative nitrogen balances. Therefore, although the nitrogen balances were not determined, it seemed that, in all probability, they were negative.

The 3-fold purpose of the present study was to note the effect of high- and low-protein intakes on (a) the nitrogen

<sup>1</sup> These data were presented by Elizabeth Lounds in partial fulfillment of the requirements for the degree of Master of Science at the University of Chicago, June, 1946.

balances, (b) the excretions of riboflavin and (c) the riboflavin test dose returns of young women.

#### EXPERIMENTAL

The subjects were 3 young women, 26 to 27 years of age, whose physical examinations showed no abnormalities. Prior to the study, the dietary histories of subjects C and E showed apparently adequate intakes of all essential nutrients. That of subject L indicated an apparently inadequate intake; both her protein and her riboflavin intake were judged to be less than adequate according to present day standards.

After a 5-day preliminary period, the subjects were maintained on weighed diets for 3 periods of 10 days each. The protein intakes were low during the first period, high during the second and low during the third. The riboflavin intakes were relatively constant throughout the study and were somewhat lower than the recommended allowances for women.

A typical day's diet on both the low- and the high-protein intakes is shown in table 1. A variety of foods was served daily and the servings were of average size in most cases. In addition, as much day-to-day variation was allowed in the kinds of fruits and vegetables served as was possible without significantly altering the daily protein and riboflavin intakes.

The diets were planned to meet the recommended daily allowances of the Food and Nutrition Board of the National Research Council ('45) for all nutrients except protein and riboflavin. Daily supplements of 700-900 mg of calcium as dicalcium phosphate wafers, 0.5 mg of thiamine<sup>2</sup> and 10 mg of niacin were given in the periods of low-protein intake (I and III) to complete the allowances and to more nearly approximate the daily intakes of period II.

The average daily intakes of different nutrients were calculated for 5 days during each period and are shown in table 2. Tables of food values compiled by Bowes and Church ('44)

<sup>2</sup> We wish to express our appreciation to Merck and Company for furnishing the synthetic vitamins used in the study.

TABLE 1

*Typical menus on low and high protein diets.*

BREAKFAST		LUNCH		DINNER	
	gm		gm		gm
Low-protein diet					
Orange juice	100	Baked potato	100	Ham	40
Blueberries	75	Asparagus	100	Yams	100
Rice flakes	20	Carrots	100	Cauliflower	75
Bread	15	Prunes	50	Head lettuce	50
Cream	100	Lettuce	20	French dressing	10
		Mayonnaise	10	Crushed pineapple	100
		Jello	100	Bread	15
		Bread	20	Lemon juice	30
		Grapefruit juice	150		
High-protein diet					
Grapes	100	Roast lamb	100	Baked trout	150
Cornflakes	25	Green beans	50	Corn	100
Bacon	12	Squash	100	Cabbage	50
Bread	45	Celery hearts	50	French dressing	10
Cream	50	Applesauce	100	Orange	100
		Bread	45	Bread	45
		Peanut butter	20	Photolyzed non-fat milk solids <sup>1</sup>	50
		Punch:			
		Grape juice	100		
		Lemon juice	50		
		Gelatin	20		

Ad libitum on both diets: sugar, butter, jelly and coffee.

<sup>1</sup> Served with sugar and vanilla as a milk-shake.

TABLE 2

*Calculated average daily intakes of different nutrients.*

PERIOD	CAL.	PRO-TEIN	FAT	CARBO-HY-DRATE	Ca	P	Fe	VIT. A	THIA-MINE	RIBO-FLA-VIN	NIA-CIN	VIT. C
		gm	gm	gm	gm	gm		I.U.	mg	mg	mg	mg
I	1508	32	60	210	1.3	2.1	9	10,390	1.4	0.9	19	162
II	2133	110	81	241	0.8	1.4	12	3,360	1.2	1.1	23	122
III	2024	28	80	298	1.1	1.7	10	13,590	1.5	0.9	18	206
Recom- mended allow- ance	2100	60			0.8		12	5,000	1.1	1.5	11	70



and by Taylor ('42) were used. That the caloric intake was undesirably low in period I was verified by weight losses in all of the subjects; this condition was corrected in periods II and III during which time usual weights were regained and maintained. The amount of iron was slightly lower than that recommended, but it seemed highly improbable that this would influence the results in a study of such short duration. Therefore, no iron supplement was given. The vitamin A intake was low in period II, but the average intake for all periods was well over the recommended allowance.

Individual food composites, consisting of one-fifth of the amount of each food eaten with the exception of bread, dry cereals and coffee, were made for 5-day periods. The bread, dry cereals and coffee were analyzed separately. Butter and cane sugar were not analyzed.

Test doses of 20  $\mu$ g of riboflavin per kilogram of body weight were given immediately before breakfast at the beginning of the study and at the end of each experimental period; this was followed by a 4-hour urine collection. On the previous day, after an identical breakfast, a 4-hour control urine specimen was collected. The breakfasts on these days were essentially the same throughout the study, although the diet as a whole was a continuation of the diet in the preceding period. The test dose returns were calculated by subtracting the amount of riboflavin excreted in the control sample from that excreted after the test dose.

Complete collections of both urine and feces were made throughout each experimental period. Twenty-four urine specimens were collected daily. Each sample was immediately preserved with hydrochloric acid and toluene and then refrigerated. Five-day fecal collections, marked with carmine, were homogenized with 95% alcohol, acidified with hydrochloric acid to a pH of 1.0 and refrigerated.

The daily urine specimens and the 5-day food and fecal composites were analyzed for nitrogen by the Kjeldahl method and for riboflavin by the microbiological method of Snell and Strong ('39) as modified by Strong and Carpenter ('42).

Daily nitrogen balances were calculated by subtracting the daily urinary excretions and average daily fecal excretions from the average daily intakes.

## RESULTS AND DISCUSSION

### *Riboflavin excretions and nitrogen balances*

The average daily riboflavin and nitrogen intakes, the corresponding fecal excretions, the daily urinary excretions and the daily nitrogen balances of the individual subjects for the first and last half of each period are shown in table 3.

On daily nitrogen intakes of approximately 5 gm in period I, subject C was in negative nitrogen balance for 9 out of 10 days and E for the entire 10 days. The balances were decidedly negative during the first part of the period. Later they were only slightly negative and by the eighth day they were in approximate equilibrium. The corresponding daily riboflavin excretions were 600  $\mu$ g or more (approximately 60% of the intake) when the nitrogen balances were strongly negative and from 200 to 350  $\mu$ g (approximately 30% of the intake) when the nitrogen balances were only slightly negative.

In period II, on nitrogen intakes of 18 and 19 gm both subjects were in positive nitrogen balance for the entire 10 days. In both cases, the greatest storage occurred during the first part of the period. Although the riboflavin intakes were somewhat higher in this period than in period I (1285 and 1450  $\mu$ g as compared to 950  $\mu$ g), the daily riboflavin excretions immediately dropped to about 100  $\mu$ g (approximately 7% of the intake).

In period III, when both the nitrogen and the riboflavin intakes of subjects C and E were comparable to those in period I, the nitrogen balances were again decidedly negative during the first part of the period and approached equilibrium in the last part. The daily riboflavin excretions were decidedly higher than those of period II. They represented almost 40% of the intake when the nitrogen balances were strongly negative and about 20% of the intake as nitrogen equilibrium was approached.

TABLE 3  
Intakes and excretions of nitrogen and riboflavin.

PERIOD	Intake	NITROGEN			Balance	RIBOFLAVIN		
		Urine	Feces	gm/day		Intake	Urinary excretion	Fecal excretion
	gm/av day	gm/day	gm/av. day	gm	gm/av. day	gm/av. day	gm/day	gm/av. day
Subject C								
Ia	4.87	7.27, 8.10, 5.70, 6.31, 3.74	0.74	— 3.14, — 3.97, — 1.57, — 2.18, + 0.39	972	738, 622, 694, 636, 226		422
Ib	4.76	4.64, 5.14, 4.93, 4.81, 4.45	0.61	— 0.49, — 0.99, — 0.78, — 0.66, — 0.30	893	249, 254, 344, 210, 193		318
IIa	17.74	7.98, 10.23, 11.78, 13.35, 12.84	0.88	+ 8.88, + 6.63, + 5.08, + 3.51, + 4.02	1237	158, 94, 101, 154, 73		442
IIb	17.68	14.03, 13.92, 10.02, 16.52, 16.29	0.87	+ 2.78, + 2.89, + 6.79, + 0.29, + 0.52	1322	93, 77, 103, 182, 114		430
IIIa	4.83	9.91, 6.11, 6.11, 5.58, 5.17	0.79	— 5.87, — 2.07, — 2.07, — 1.54, — 1.13	1016	494, 507, 250, 379, 317		601
IIIb	4.82	4.55, 4.69, 4.34, 3.98, 4.08	0.81	— 0.54, — 0.68, — 0.33, + 0.03, — 0.07	859	308, 279, 128, 124, 196		473
Subject E								
Ia	5.20	7.22, 6.76, 5.91 5.85, 5.85	1.08	— 3.10, — 2.64, — 1.79, — 1.73, — 1.73	999	722, 695, 577, 615, 392		624
Ib	4.71	4.57, 4.72, 4.56, 4.17, 3.97	1.16	— 1.02, — 1.17, — 1.01, — 0.62, — 0.42	957	328, 242, 342, 339, 278		949
IIa	18.63	8.37, 11.96, 12.83, 15.58, 14.62	1.64	+ 8.62, + 5.03, + 4.16, + 1.11, + 2.37	1519	201, 103, 101, 149, 97		883
IIb	19.36	12.79, 15.83, 15.48, 17.35, 16.70	1.25	+ 5.32, + 2.28, + 2.63, + 0.76, + 1.41	1383	172, 101, 130, 126, 147		765
IIIa	4.98	9.67, 6.21, 5.16, 5.55, 5.14	1.51	— 6.20, — 3.74, — 1.69, — 2.08, — 1.67	945	316, 379, 234, 238, 263		877
IIIb	4.90	4.35, 4.77, 4.85, 4.36, 4.25	1.05	— 0.50, — 0.92, — 1.00, — 0.51, — 0.40	935	310, 215, 185, 131, 238		825
Subject L								
Ia	5.85	4.51, 5.02, 4.78, 3.54, 4.02	1.18	+ 0.16, — 0.35, — 0.11, + 1.13, + 0.65	1060	391, 396, 358, 223, 220		894
Ib	5.19	3.77, 1.38, 3.45,		+ 0.07, + 2.46 + 0.39		915, 61, 189		

Although the nitrogen losses of both subjects were greater in period III than in period I, the riboflavin excretions were less. This can probably be explained on the basis of the diets prior to the study. Both subjects had regularly consumed a diet which included at least  $1\frac{1}{2}$  pints of milk daily as well as generous amounts of meat, eggs, vegetables and fruit. Their daily riboflavin intakes on such diets were obviously considerably more than 1000  $\mu\text{g}$ . Therefore, they excreted relatively large amounts of riboflavin during the first few days as they adjusted to the lower intakes.

The results obtained on subject L show the same general pattern as those on subjects C and E, but at a different level. Her nitrogen balances were for the most part, slightly positive during period I on the low nitrogen intake. This indicated that the nitrogen content of her diet prior to the experiment had probably been quite similar to that of her diet in period I. Additional evidence of this was obtained from her dietary history which showed that her eating habits had been both irregular and erratic; for the most part she had subsisted on snacks taken at odd intervals rather than regular meals. Still further evidence of her depleted state is shown by the magnitude of her nitrogen balances in period II on the high-protein diet. She stored over twice as much nitrogen in that period as subjects C and E. Following these strongly positive balances, her balances were negative in period III, although to a lesser degree than those of subjects C and E. The daily riboflavin excretions of subject L followed the same general trend as those of subjects C and E, but also to a lesser degree.

Correlation coefficients,<sup>3</sup> calculated between the daily nitrogen balances and riboflavin excretions were found to be highly significant for all 3 subjects. They were  $-0.69$ ,  $-0.57$  and  $-0.69$  for subjects C, E and L, respectively.

This shows that in human subjects as in animals an inverse relationship exists between urinary riboflavin excretions and nitrogen balances. This relationship seems to hold, regardless

<sup>3</sup> Correlation coefficients were calculated according to the Product-Moment formula.

of whether the negative nitrogen balances are brought about by a low-protein diet or by other factors (Andrea et al., '46; Roderuck et al., '46). It also seems to be independent of small changes in body weight. In the present study, weight losses of 1 to 2½ kg occurred in all 3 subjects during period I and gains of approximately 1 kg were made by 2 of the subjects in period II. In period III, however, only 1 subject showed a change in weight.

It is not surprising that riboflavin and labile protein are closely related since the main storage organ of both is the liver (Luck, '36; Kosterlitz and Campbell, '45). Although the kidney also has a high concentration of riboflavin (Taylor et al., '42; Mitchell and Isbell, '42), Czaczkcs and Guggenheim ('46) report that it is unable to store this vitamin.

That the riboflavin content of the livers of animals is dependent on protein intakes rather than on riboflavin intakes has been demonstrated in several laboratories (Sarett and Perlzweig '43; Unna et al., '44; Reisen et al., '46; Czaczkcs and Guggenheim, '46; Wright and Skeggs, '46). The relatively high riboflavin excretions during periods of negative nitrogen balance may be the result of the inability of the liver to store riboflavin or the release of previously stored riboflavin or both. The findings of Czaczkcs and Guggenheim ('46), however, indicate that the low riboflavin excretions during periods of positive nitrogen balance cannot be due to storage of the vitamin by the liver. These authors believe them to be due to an increased dietary requirement which in turn, results from a decreased intestinal synthesis since both the fecal riboflavin excretion and the number of viable bacteria in the feces of their animals on the high-protein diet were less than those of animals on a normal diet.

In the present study, however, no such decrease in fecal riboflavin occurred during the period of high-protein intake (table 3). Although these data cannot be interpreted as evidence that there was no change in the rate of intestinal synthesis, they do make it seem questionable and would lend support to the possibility that other factors may have been

operating, in the case of these subjects, to promote liver storage or to increase the requirement of the vitamin.

### *Riboflavin test dose returns*

Since the amount of riboflavin excreted in the urine varied with the coexistent nitrogen metabolism, it would seem logical to expect the returns of riboflavin test doses to show a similar variation. Sarett et al. ('42) found such to be the case in dogs. In the present study the returns of subjects C and L were lower after periods of positive nitrogen balance or approximate equilibrium than after periods of negative nitrogen balance (table 4). Those of subject E, however, increased

TABLE 4

*Per cent return of riboflavin test doses.*

SUB- JECT	PRE PERIOD			PERIOD I			PERIOD II			PERIOD III		
	Test dose	4-hour return <sup>1</sup>		Test dose	4 hour return <sup>1</sup>		Test dose	4 hour return <sup>1</sup>		Test dose	4-hour return <sup>1</sup>	
	$\mu g$	$\mu g$	%	$\mu g$	$\mu g$	%	$\mu g$	$\mu g$	%	$\mu g$	$\mu g$	%
C	1000	188	19	990	322	33	1001	224	22	986	366	37
E	1100	180	16	1089	207	19	1086	288	27	1087	332	31
L	1200	49	4	1238	54	4	1274	64	5	1256	113	9

<sup>1</sup> Corrected for the amount excreted during the 4-hour control period.

progressively throughout the study and showed no relationship to her nitrogen balances. As a result no definite conclusions can be drawn from these data as to the effect of nitrogen balances on test dose returns. However, since 2 of the subjects showed the same inverse relationship as was found by Sarett et al. ('42), on dogs, and since Czaczkes and Guggenheim ('46) reported that rats on a low-protein diet lacked the ability to retain riboflavin, it would seem advisable to give consideration to the nitrogen metabolism when interpreting the results of riboflavin test dose returns.

### SUMMARY AND CONCLUSIONS

Three young women were maintained on weighed diets for 3 periods of 10 days each. The daily nitrogen intakes were

approximately 5 gm during the first period, 19 gm during the second and 5 gm during the third. The daily riboflavin intakes were approximately 1000  $\mu$ g during the first period, 1200 to 1400  $\mu$ g during the second and 1000  $\mu$ g during the third. Urinary riboflavin and nitrogen excretions were determined daily; the amounts in foods and feces were determined on individual 5-day composites. Daily nitrogen balances were calculated by subtracting daily urinary excretions and average daily fecal excretions from average daily intakes.

The majority of the nitrogen balances were negative during the periods of low-nitrogen intake and all were positive during the period of high-nitrogen intake.

Daily riboflavin excretions varied inversely with the co-existent nitrogen balances. They represented from 40 to 60% of the riboflavin intake when the nitrogen balances were decidedly negative and approximately 7% of the intake when the nitrogen balances were strongly positive.

The returns of riboflavin test doses given at the end of each period showed the same inverse relationship with the nitrogen balances in 2 of the subjects.

Fecal riboflavin excretions did not appear to be influenced by the alterations in the composition of the diet and showed no relationship to the nitrogen balances.

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# GROWTH AND LONGEVITY OF RATS FED OMNIVOROUS AND VEGETARIAN DIETS <sup>1</sup>

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THREE FIGURES

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Comparative studies of the effects of omnivorous and purely vegetarian diets on the growth and longevity of rats seem of interest as the increasing population of the world tends to compel the use of a larger proportion of foods of vegetable origin in the human diet and the view is widely held that a vegetarian diet promotes health and longevity. Slonaker ('12) determined the effect on rats of a diet which he called a strictly vegetarian diet but this diet included bread, cookies and other items which introduced small amounts of foods of animal origin, such as milk, butter and eggs. The growth of Slonaker's rats on the so-called strictly vegetarian diet was nevertheless impaired and they lived only about half as long (555 days) as control rats (1020 days) which were fed the same diet with the addition of meat and other foods of animal origin. Extensive comparative studies of the effects of purely vegetarian and omnivorous diets were made on rats by Wu and his associates (Wu and Wu, '28; Wu, Wan and Chen, '32; Chang, Wu and Chen, '41; and Chen, Chang and Luo, '41). The growth of rats on the best (economical) purely vegetarian diet that Wu and his associates devised was inferior to growth on their omnivorous diets but the vegetarians

<sup>1</sup> This study was aided by a grant from Swift and Co., Chicago.

were found to live as long or longer than the omnivorous rats (Chang, Wu and Chen). That is, the male vegetarians did not live significantly longer (540.1 days) than the omnivorous males (526.7 days) but the vegetarian females lived significantly longer (611.1 days) than the omnivorous females (526.9 days). The longer life span of the vegetarian females was attributed to their retarded growth but this does not explain why the vegetarian males failed to live significantly longer than the omnivorous males. The data of Chang, Wu and Chen show that their omnivorous females did not live longer than the omnivorous males and this is contrary to general findings. It therefore seems that, instead of the life span of the females having been lengthened by a vegetarian diet, the life span of the females on the omnivorous diets was somehow shortened. A further study of the effect of omnivorous and vegetarian diets on longevity and incidentally, on growth therefore appeared to be desirable.

#### METHODS

As indicated in a preceding paper (Carlson and Hoelzel, '46) we included the use of omnivorous diets and a purely vegetarian diet in a study of the effect of intermittent fasting on the longevity of rats. In that study, 45 Wistar rats (19 males and 26 females) were fed our basic omnivorous diet (diet 1) and 46 rats (21 males and 25 females) were fed the purely vegetarian diet (diet 4). Besides this 46 rats were fed diet 1 with 10% added cellulosic bulk-formers (diets 2 and 3) but the details concerning the effects of these and other bulky omnivorous diets will be reported in a separate paper. Diets 1 and 4 were described in the preceding paper and the composition is included again in table 1. Approximately equal numbers of the rats fed diet 1 and 4 were fed these diets ad libitum or were fasted 1 day in 4, 1 day in 3 or 1 day in 2. Littermates of 7 males and 18 females fed diet 1 were included in corresponding groups fed diet 4 and littermate rats were also included in the different groups on the same diet.

In a second longevity study on Wistar rats, diet 1 was again used as the basic omnivorous diet but, in place of diet 4, a vegetarian self-selection diet (diet 6, table 1) was used. Each item in diet 6 was presented in a separate container or in a separate compartment of a larger container. The peas and soy beans included in diet 6 were first soaked 18 hours in water. Most of the rats in this study were however fed diet 1

TABLE 1  
*Composition of diets.*

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Diet 1 — 61.5% dried "whole veal," 3% veal bone meal, 31% corn starch, 2% dried brewer's yeast, 1.5% inorganic salt mixture and 1% cod liver oil. Supplemented by lettuce ad libitum.
Diet 4 — 50% stone-ground whole wheat flour, <sup>2</sup> 10% peanut flour, 7% wheat gluten flour, 7% lima bean flour, 7% linseed meal, 7% corn gluten meal, 5% alfalfa leaf meal, 5% brewer's yeast, and 2% NaCl. Supplemented by lettuce ad libitum.
Diet 6 — Self-selection from corn (whole kernels), wheat (whole grain), pearled barley, rolled oats, sunflower seeds, peanuts, green peas, soy beans, defatted corn germ meal, <sup>3</sup> defatted wheat germ meal, <sup>3</sup> brewer's yeast, alfalfa leaf meal and NaCl. Supplemented by lettuce ad libitum.
Diet 6C — Diet 6 supplemented by celery cabbage as well as by lettuce ad libitum.
Diet 6CC — Diet 6 supplemented only by celery cabbage ad libitum.

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<sup>1</sup> Specially prepared for us by Swift and Co., Chicago, and previously described (Carlson and Hoelzel, '46).

<sup>2</sup> Obtained from Elam Mills, Chicago.

<sup>3</sup> Obtained from VioBin Corp., Monticello, Ill.

with added cellulosic or other bulk-formers (diets 3, 5, 7, 8, 9, 10 and 11) but, as already stated, their specific effects will be the subject of a separate report. Other differences between the second and the first longevity study were as follows: The first aim was to use rats likely to be more uniform genetically than the rats used in our first study. This objective was presumably attained by using 18 litters obtained from 4 females and 2 males (father and son) instead of 17 litters obtained from 16 females mated with 6 males as in the first longevity study. No first litters were used and, to promote

pre-experimental nutritional uniformity, litters of more than 8 rats were reduced to 8, 7 or 6 rats. The 18 litters provided 115 rats (57 males and 58 females), including rats born in each of the 4 seasons. To make it possible to determine the influence of the different diets at the earliest possible ages, successive litters from the same mothers were raised from the time when they first began to eat on the different experimental diets. The mothers however were left with their litters until the litters were 35 days old but the mothers were fed supplementary food separately to keep them in as uniform a condition as practical for further breeding. The sires were kept on diet 1. Five litters, including 17 males and 16 females, were started on diet 1. Eleven litters were started on diet 1 with 10% added bulk-formers (diets 3, 5, 9 and 10). Only 2 litters, including 6 males and 7 females, were started on diet 6 before a deterioration of some of the rats on this diet suggested that the starting of more rats on this diet was not warranted. The original intention was to keep about half of the rats on the same diet throughout life and to transfer the other half to 1 of the other diets when the rats became 72 days old or older. Thus 7 of the original 13 vegetarians were transferred to omnivorous diets before they became 200 days old and, because of the deterioration of some of the remaining 6, all of them were placed on omnivorous diets by the time they became 345 days old. Nevertheless 10 rats (5 males and 5 females) started on omnivorous diets were placed on diet 6 after they became 72 to 200 days old and were kept on this diet until they died. Seventy of the rats in this study were fed the diets *ad libitum* and 45 were fasted 1 day in 3 after they became 100 to 200 days old.

In addition to observations on the growth of rats made during the 2 longevity studies, short-term growth studies were made on other Wistar rats and on Sprague-Dawley rats. In these studies, diet 1 and the Sherman and Campbell diet (containing 1 part whole milk powder and 2 parts whole wheat flour with 1.33% added NaCl) plus lettuce *ad libitum* daily were used

as the omnivorous diets and diets 6, 6C and 6CC (table 1) were used as the vegetarian diets.

## RESULTS

### *First longevity study*

Figure 1 (first study) shows that the rats fed our basic omnivorous diet (diet 1) ad libitum attained weights about 30% (females) to 35% (males) greater than the average

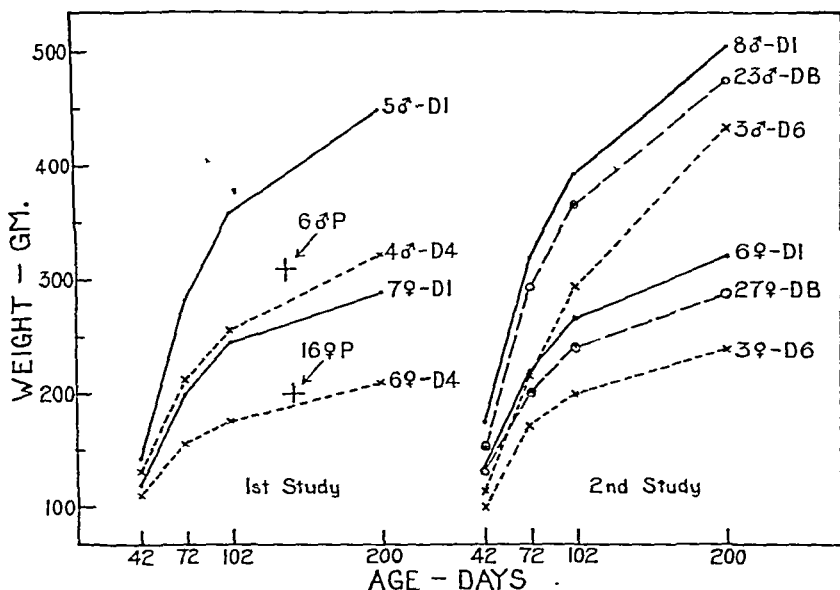


Fig. 1 Composite growth curves of rats fed omnivorous diets (D1 and DB) and vegetarian diets (D4 and D6) in 2 longevity studies. D1 = diet 1, DB = diet 1 with 10% added bulk-formers, D4 = diet 4 and D6 = diet 6. The crosses between the growth curves of the rats in the first study indicate the average weights and ages of the parent rats when they were received from The Wistar Institute.

weights of the parent rats when they were received from The Wistar Institute. On the other hand, the rats fed the vegetarian diet (diet 4), although fed this diet exclusively only after they became 42 days old, weighed less than the parent rats at similar ages. The average maximum weights attained

TABLE 2

*Maximum weights, femoral lengths and ages at death of rats fed an omnivorous diet (diet 1) and rats fed vegetarian diets (diets 4 and 6), ad libitum or with intermittent fasting, during 2 longevity studies.*

DIET	REGIMEN	NO. OF RATS	MAXIMUM WEIGHT <sup>1</sup> gm	FEMORAL LENGTH <sup>1</sup> mm	AGE AT DEATH <sup>1</sup> Days
<i>Males — First longevity study</i>					
1	Fed ad libitum	5	591 ± 212	39.0 ± 1.5	633 ± 123
4	Fed ad libitum	5	354 ± 59	38.4 ± 0.5	595 ± 146
1	Fasted 1 day in 4	5	493 ± 107	39.0 ± 1.2	660 ± 91
4	Fasted 1 day in 4	5	350 ± 60	38.4 ± 1.1	685 ± 238
1	Fasted 1 day in 3	4	379 ± 96	38.4 ± 1.2	649 ± 225
4	Fasted 1 day in 3	6	318 ± 62	37.2 ± 1.7	637 ± 214
1	Fasted 1 day in 2	5	348 ± 31	37.0 ± 0.7	691 ± 112
4	Fasted 1 day in 2	5	250 ± 57	34.9 ± 1.1	607 ± 199
1	All regimens	19	453 ± 120	38.4 ± 1.1	659 ± 128
4	All regimens	21	323 ± 56	37.2 ± 1.1	631 ± 187
<i>Littermate males only</i>					
1	All regimens	7	363 ± 85	37.7 ± 0.8	629 ± 127
4	All regimens	7	264 ± 27	35.7 ± 1.2	572 ± 170
1	Controls <sup>2</sup>	12	474 ± 179	38.5 ± 1.1	600 ± 105
1	Fasted <sup>2</sup>	12	394 ± 105	38.0 ± 1.5	680 ± 154
4	Controls <sup>2</sup>	13	348 ± 67	38.0 ± 1.4	628 ± 180
4	Fasted <sup>2</sup>	13	296 ± 66	36.5 ± 1.8	674 ± 191
<i>Females — First longevity study</i>					
1	Fed ad libitum	7	456 ± 187	34.7 ± 0.2	659 ± 125
4	Fed ad libitum	6	236 ± 33	33.9 ± 0.8	619 ± 232
1	Fasted 1 day in 4	7	343 ± 38	35.1 ± 0.8	685 ± 101
4	Fasted 1 day in 4	7	215 ± 50	34.3 ± 0.8	545 ± 236
1	Fasted 1 day in 3	7	351 ± 60	34.9 ± 1.1	834 ± 138
4	Fasted 1 day in 3	7	239 ± 43	34.7 ± 0.6	762 ± 190
1	Fasted 1 day in 2	5	280 ± 56	34.5 ± 0.8	754 ± 103
4	Fasted 1 day in 2	5	200 ± 21	32.6 ± 0.7	613 ± 214
1	All regimens	26	359 ± 100	34.8 ± 0.8	733 ± 112
4	All regimens	25	223 ± 34	33.9 ± 0.7	650 ± 204

TABLE 2 (continued)

DIET	REGIMEN	NO. OF RATS	MAXIMUM WEIGHT <sup>1</sup> gm	FEMORAL LENGTH <sup>1</sup> mm	AGE AT DEATH <sup>1</sup> days
<i>Littermate females only</i>					
1	All regimens	18	333 ± 58	34.7 ± 0.8	723 ± 154
4	All regimens	18	214 ± 33	33.8 ± 1.0	630 ± 214
1	Controls <sup>2</sup>	18	427 ± 165	34.8 ± 0.4	702 ± 126
1	Fasted <sup>2</sup>	18	335 ± 59	35.0 ± 0.8	753 ± 131
4	Controls <sup>2</sup>	16	226 ± 41	34.2 ± 0.7	619 ± 248
4	Fasted <sup>2</sup>	16	230 ± 44	33.9 ± 1.3	645 ± 152
<i>Males — Second longevity study</i>					
1	Fed ad libitum	4	639 ± 169	39.3 ± 0.6	588 ± 96
6-1 <sup>3</sup>	Fed ad libitum	1	570	37.8	511
1-6 <sup>4</sup>	Fed ad libitum	1	524	40.9	564
1	Fasted 1 day in 3	4	534 ± 86	38.2 ± 1.1	589 ± 206
6-1 <sup>3</sup>	Fasted 1 day in 3	0			
1-6 <sup>4</sup>	Fasted 1 day in 3	1	458	38.3	470
<i>Females — Second longevity study</i>					
1	Fed ad libitum	3	551 ± 72	33.2 ± 0.5	701 ± 129
6-1 <sup>3</sup>	Fed ad libitum	1	244	32.1	379
1-6 <sup>4</sup>	Fed ad libitum	1	362	34.3	585
1	Fasted 1 day in 3	3	357 ± 63	34.1 ± 1.5	779 ± 298
6-1 <sup>3</sup>	Fasted 1 day in 3	0			
1-6 <sup>4</sup>	Fasted 1 day in 3	1	330	32.9	835

<sup>1</sup> Mean and standard deviation.<sup>2</sup> The controls include rats fed ad libitum and rats fasted 1 day in 4 or 3 but all fasted control rats fasted less than their fasted littermates.<sup>3</sup> Diet 6 fed early in life and diet 1 fed later in life.<sup>4</sup> Diet 1 fed early in life and diet 6 fed later in life.

on the 2 diets and 4 regimens are presented in table 2. The greatest individual weights attained by rats fed the diets ad libitum were: male on diet 1, 870 gm, male on diet 4, 450 gm, female (with mammary tumor) on diet 1, 855 gm, and female, (with gastric and uterine tumors) on diet 4, 294 gm. Figure 2 and table 2 show that the weights attained by the intermittently fasted rats were inversely proportional to the



amount of fasting. The sizes attained, as indicated by femoral length at death (table 2), were more uniform than the weights. The ages attained (fig. 2 and table 2) were, on the average, greater in rats fed diet 1 than in rats fed diet 4 and greater in fasted rats than in rats fed ad libitum. Mammary tumors developed in 8 (31%) of the females on diet 1 but only in 2 (8%) of the females on diet 4. Moreover, the mammary tumors developing in females fed diet 1 were much larger than those in females fed diet 4—the largest tumor in a female fed diet 1 (462 gm) weighed much more than the heaviest female (with tumors) fed diet 4 (294 gm).

### *Second longevity study*

Figure 1 (second study) shows that the feeding of the different experimental diets earlier in life than in the first study produced considerable divergences in the weights already at the age of 42 days. The omnivorous rats weighed more and the vegetarians less at 42 days than the rats in the corresponding groups in the first study but the vegetarians as well as the omnivores in the second study attained greater weights, by the time they became 200 days old, than the corresponding rats in the first study. In fact, the males fed the vegetarian self-selection diet (diet 6) ad libitum gained more weight between the ages of 72 days and 200 days than the rats in this study fed diet 1 or diet 1 plus bulk-formers ad libitum (fig. 1). However, the rats started on diet 6 that were fasted 1 day in 3 did not seem to fare as well as rats fed diet 4 and fasted 1 day in 3 in the first longevity study. The rats fed diet 6 ad libitum also began to lag considerably in growth, in comparison with the rats on the omnivorous diets, after they became 200 days old. Early deaths among the intermittently fasted vegetarians seemed likely. Hence, all of the rats started on diet 6 were transferred to omnivorous diets. Rapid improvement in the deteriorated rats followed. Rats that were started on the omnivorous diets and transferred to diet 6 when they were between 72 and 200 days old tolerated fasting

1 day in 3 better. Table 2 includes data on rats in the second longevity study that were fed diet 1 throughout life and rats that were fed diet 6 before or after diet 1. The data indicate that the rats fed diet 6 early in life or late in life did not grow as well or live as long as the rats fed diet 1 throughout life. The maximum weights attained by individual rats in the second longevity study were: male on diet 1, 890 gm, male on diet 6 (early in life), 570 gm, female (with mammary tumor) on diet 1, 574 gm, and female (with ovarian tumor) on diet 6

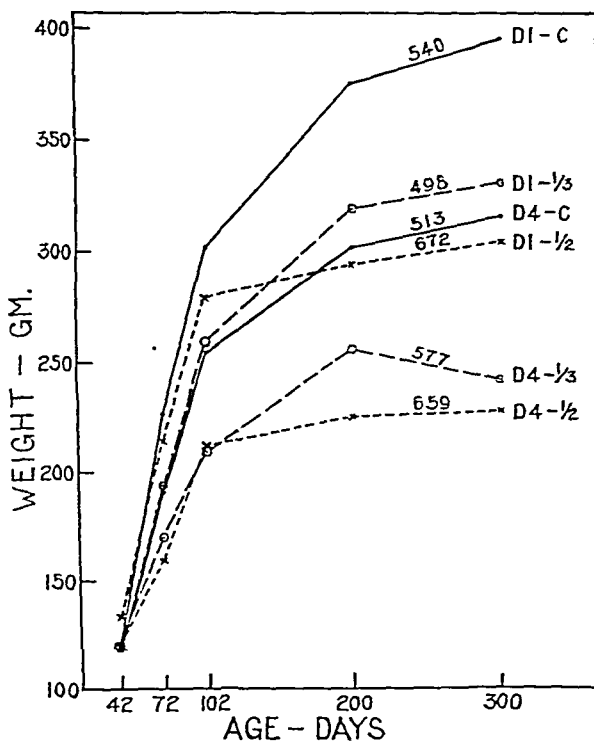


Fig. 2 Composite growth curves of 2 littermate trios of male rats fed the basic omnivorous diet (D1) and 2 trios fed the vegetarian diet (D4) with 1 of the littermates in each trio fed the diet ad libitum (control or C) 1 fasted 1 day in 3 ( $\frac{1}{3}$ ) and the third fasted 1 day in 2 ( $\frac{1}{2}$ ). The trios on D1, however, were not littermates of the trios on D4. The figures on the curves indicate the average age attained by the 2 rats on each regimen. The decrease between the ages of 200 and 300 days in the average weight of the 2 rats on D4 and fasting 1 day in 3 was due to the early deterioration of 1 of these rats.

(late in life), 362 gm. Four of the 6 females fed diet 1 throughout life developed mammary tumors; none of the 3 females fed diet 6 early or late in life and diet 1 late or early in life developed such tumors.

### *Short-term growth studies*

Alternate litters raised from the same (Wistar or Sprague-Dawley) rats on either diet 1 or the Sherman and Campbell diet plus lettuce daily showed that the weights attained on the Sherman and Campbell diet were uniformly lower than

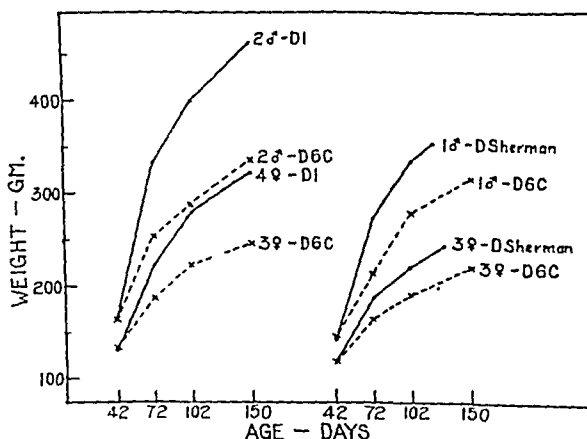


Fig. 3 Composite growth curves of rats fed the basic omnivorous diet (D1) with littermates fed the vegetarian self-selection diet supplemented by celery cabbage as well as by lettuce (D6C) and rats fed the Sherman and Campbell diet (D Sherman) with littermates fed D6C.

weights attained on our basic omnivorous diet. The relative growth-promoting powers of these 2 omnivorous diets can be inferred from the growth curves in figure 3 as the effect on littermate rats was similar to the depicted effects on rats belonging to different litters. A comparison of the growth curves in figure 3 with the growth curves of the rats represented in figure 1 also shows that the inclusion of celery cabbage in the vegetarian diet did not significantly improve growth in comparison with growth on omnivorous diets.

## DISCUSSION

The great weights attained by some of the rats on our basic omnivorous diet (diet 1) seem explainable by the nature of the diet. This diet was designed to supply meat from young animals (calves) as the chief source of protein and to include enough protein to meet the assumed requirements of intermittently fasted rats. The dried meat ("whole veal"), as originally prepared for us by Swift and Company, contained about 50% protein and 40% fat. A diet containing about 35% protein seemed to be needed and the fat in the "whole veal" made the diet practically a paste diet including about 28% fat. In short, diet 1 became a diet rich in animal protein and fat like the growth-promoting paste diet which is included in the Anderson and Smith ('32) diet. Moreover, although our aim was to use mainly natural foods, we used corn starch rather than whole wheat flour as the source of carbohydrate in diet 1 to avoid introducing more vegetable protein into this diet than necessary. As a consequence, diet 1 became a concentrated diet containing a minimum of roughage. Diet 1 undoubtedly also appealed considerably to the appetite of the rats and growth was approximately as rapid as the growth observed by others on the Anderson and Smith diet (Dunn, Murphy and Rockland, '47).

The vegetarian diet used in our first longevity study (diet 4) was designed to make whole wheat flour the chief component. The other components served mainly to increase the protein content of the diet. Diet 4 contained about 30% protein and 8% fat but because of the difference in fat content between diet 4 and diet 1, diet 4 supplied more protein than diet 1 on an isocaloric basis. Diet 4, incidentally, contained much more roughage than diet 1 and our bulky omnivorous diets were designed to determine the effect of the bulkiness of vegetarian diets on growth and longevity. Figure 1 (second study) indicates that less than half of the lower weight of vegetarians is accounted for by the bulkiness or non-nutritive fraction of a vegetarian diet like diet 6. The relatively poor growth of rats on a vegetarian diet is therefore mainly due to

the unsatisfactory nature of the nutritive components of the diet.

The higher general weight levels attained by rats in the second life span study were evidently mainly due to the use of only second and later litters and limiting the size of the litters. Seasonal differences in the growth rates of rats on diet 1 and diet 6 were found to explain the relatively more rapid growth of the vegetarian males in the second longevity study between the ages of 72 days and 200 days. That is, the vegetarians fared best in summer while the rats on diet 1 grew most rapidly in cooler seasons. Our rat quarters were not air-conditioned and the period when the vegetarians were between 72 and 200 days old included the summer period.

The great variations in the maximum weights of the rats fed diet 1 ad libitum (indicated by the standard deviations in table 2) are only partly explainable. In the first longevity study, the variations in maximum weights were largely due to variations in initial weights. The ages attained before growth ceased also varied more in the first study than in the second. The failure of the males in the second longevity study to attain more uniform maximum weights suggested that a rich diet like diet 1 fed ad libitum may have some deteriorating effect on successive generations but a further study of this possibility will be necessary before definite conclusions can be drawn. The maximum weights of the females on diet 1 varied mainly because of differences in the occurrence of and size attained by mammary tumors. It became evident in the second longevity study that the size attained by the mammary tumors depended partly on their location. Mammary tumors which interfered with eating, defecating or urinating were usually chewed off by the rats.

The great variations in the life span of the rats, particularly of the vegetarians fed diet 4 (table 2) naturally raised the question whether the life spans of the rats fed diet 1 were significantly greater than the life spans of the rats fed diet 4. The data on littermate males alone and littermate females alone did not show a significant difference but the combined

littermate male and female data showed that the omnivorous rats lived significantly longer ( $P < 5\%$ ). However, the greater variations in the life spans of the vegetarian rats furnish a basis for the widely held impression that vegetarianism prolongs life. That is, although the vegetarian rats, on the average, died sooner than the omnivorous rats, 4 vegetarians became over 1000 days old while only 1 omnivorous rat became over 1000 days old. Moreover, the ageing vegetarians looked younger than the ageing omnivorous rats — the vegetarians did not develop skin lesions and alopecia like most of the old omnivorous rats.

The effect of intermittent fasting on the life spans also indicated that the omnivorous diet was superior to the vegetarian diet. The combined data on 12 pairs of littermate males and 18 pairs of littermate females fed diet 1 (table 2) showed that the fasted littermates lived significantly longer than their controls (average, 62.8 days longer —  $P < 2\%$ ). In contrast to this, 13 pairs of littermate males together with 16 pairs of littermate females fed diet 4 failed to show that the fasted littermates lived significantly longer (average, only 34.6 days longer —  $P > 50\%$ ). In our preceding report ('46),<sup>4</sup> the view was expressed that fasting 1 day in 3 was the optimum amount of fasting for rats. Fasting 1 day in 4 did not seem to be of significant value. Fasting 1 day in 2 on the other hand seemed to be too much fasting. Figure 2, for example, shows that the growth of both omnivorous and vegetarian rats fasted 1 day in 2 tended to be stunted after they became about 100 days old and an amount of fasting or food restriction which stunts growth must be regarded as too much fasting or too much food restriction even if the life span (or mere existence) is thus prolonged. Fasting 1 day in 3 did not significantly stunt growth (size as indicated by femoral length) although it limited weight and prolonged life. Twelve of our rats fed diet 1 ad libitum or that were fasted only 1 day in 4 had littermates fed the same diet and fasted 1 day in 3. The littermates fasted 1 day in 3 lived significantly longer (average, 99 days longer —

$P < 2\%$ ). Similar data on 10 pairs of littermates fed diet 4 did not show that the littermates fasted 1 day in 3 lived significantly longer. Actually the vegetarians fasted 1 day in 3 lived, on the average, 105 days longer than their littermate controls and this suggests that fasting 1 day in 3 was also the optimum amount of fasting for the vegetarians but the variations in the life spans of the vegetarians were so great that a much larger number of rats would have been necessary to show that even an average of 105 days longer life represented a significant increase in the life span ( $P > 50\%$ ).

The data secured during our second longevity study were too meager to be treated statistically but it can be seen (table 2) that the results were similar to those obtained during our first longevity study. The results can therefore be regarded as additional evidence that a purely vegetarian diet tends to shorten life and that fasting 1 day in 3 tends to prolong life. This will become clearer when the results of feeding diet 1 with added bulk are reported.

A possible criticism of our longevity studies is that the vegetarian diets were not supplemented by a suitable fresh leafy vegetable. Wu and Wu ('28) found that the kinds and proportions of grains and legumes in vegetarian diets had no significant influence on the adequacy of such diets but that the type of fresh leafy vegetable supplement used was of considerable importance. Lettuce was found to be a poor leafy vegetable supplement to a vegetarian diet. Wu and his associates used colza or Chinese "small cabbage" in their extensive studies. Colza and Chinese "small cabbage" were not obtainable in local markets. We supplied the rats with various other greens besides lettuce from time to time but the rats preferred lettuce. Hence, we fed mainly lettuce as the supplement to all diets. However, after Adolph ('44) stressed the value of celery cabbage as a supplement to a diet of grains and legumes we tried this in short-term growth studies on Wistar and Sprague-Dawley rats. The results have not convinced us that the celery cabbage obtainable in local markets improves the growth of rats or would be likely to signifi-

cantly influence the life span. The rats preferred lettuce to celery cabbage when both were provided and also did not eat much celery cabbage when no lettuce was provided.

The observed results on the rats confirm the personal experiences of 1 of us (H) that a purely vegetarian diet (chiefly a fruit and nut diet) was unsatisfactory, that fasting was more easily borne and apparently of more benefit when an omnivorous diet was used and that fasting 1 day in 2 was too much fasting regardless of diet.

### SUMMARY

The growth of rats fed omnivorous and vegetarian diets was observed during 2 longevity studies and during additional short-term studies.

Two of 9 male Wistar rats attained weights of 870 and 890 gm, respectively, on an omnivorous diet including about 35% protein (chiefly meat protein) and 28% fat.

Rats fed purely vegetarian diets were stunted in growth.

Rats fed an omnivorous diet lived significantly longer than rats fed a vegetarian diet. Moreover, the life span was more uniformly prolonged by the intermittent fasting of rats fed an omnivorous diet than in rats fed a vegetarian diet.

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# EXPERIMENTS WITH DELAYED SUPPLEMENTATION OF INCOMPLETE AMINO ACID MIXTURES<sup>1</sup>

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FOUR FIGURES

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The optimal composition of amino acid mixtures for therapeutic uses is still under investigation. The logical way to determine the optimal composition appears to be to calculate statistically from the analysis of a so-called "normal" diet the quality and relative quantity of the amino acids present in the average daily protein intake (Block, '43; Mitchell and Block, '46). An amino acid mixture which duplicates the composition of such an average daily protein supply could *a priori* be regarded as satisfactory. Against such a conclusion, however, serious objections must be raised.

The dietary proteins are digested slowly, and therefore the absorption of amino acids takes place gradually, according to the rate of enzymatic liberation. On the other hand, after intravenous injection of amino acid mixtures, the organism is suddenly flooded with these compounds; on feeding of amino acid mixtures differences in rates of intestinal absorption and the rate of renal clearance of amino acids have to be considered (Chase and Lewis, '34; Goettŝch et al., '44; Eaton and

<sup>1</sup> The data of this paper were taken from a thesis submitted by the author to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>2</sup> With the technical assistance of Leslie E. Geiger.

- WU, H., AND D. Y. WU 1928 Growth of rats on vegetarian diets. Chinese J. Physiol., 2: 173.
- WU, H., S. WAN AND T. T. CHEN 1932 Growth and reproduction of rats on an improved stock diet. Chinese J. Physiol., 6: 295.

the missing building stones certain of the components of the incomplete diet would not now be available and, therefore, the animals would not show any growth.

#### METHODS

The amino acid mixture used in the experiments with tryptophane was prepared by hydrolysis of mackerel protein. This protein, as was shown by the author in collaboration with Deuel and others ('46), has a higher biological value than casein. The protein was hydrolyzed with sulfuric acid at 15-pound pressure for 8 hours. After careful removal of the sulphuric acid by barium hydroxide, the solution was concentrated under reduced pressure to a total solid content of 15%. Since the tryptophane originally present in the protein was destroyed during the acid hydrolysis, the solution prepared by this procedure was an incomplete amino acid mixture. This solution was then spray dried. When fed to rats of 40-50 gm body weight, at a level of 3% N as the sole source of protein, it did not promote growth, which would presumably indicate the absence of an essential amino acid. This incomplete mixture, when supplemented with 1% *l*(-) tryptophane, had a normal growth-promoting activity evidenced by an average daily growth of 4-5 gm.

The composition of the various diets used in these experiments is given in table 1.

For the experiments the pure bred Sprague-Dawley rats were placed in individual cages provided with wire mesh bottoms. The main difficulty encountered was with respect to determination of the daily food intake, due to the habit of some rats of spilling the feed. The following satisfactory arrangement was finally devised: Ointment jars of 40 mm diameter and 40 mm height with a metal screw cap having a circular opening of 25 mm diameter, were employed as food containers. The food was placed in the jar and covered with a piece of wire screen (1 × 1 cm mesh). The jar was then placed in a glass dish of 110 mm diameter and 60 mm height.

Dotty, '41). It is possible, therefore, that even in cases where amino acid mixtures of optimal composition are fed, they may never be present in such relative optimal concentrations simultaneously in the tissues as required for protein synthesis.

The fate of such amino acid mixtures, which by absolute or relative lack of certain constituents were either not at all or only partially utilized for protein synthesis, is, therefore, worthy of investigation. Three possibilities were considered: (1) that the amino acids can be stored in the body as such, representing thereby a pool from which protein synthesis can proceed as soon as the lacking building stones are supplemented; (2) that they are stored in the form of osmotically less active peptides; and (3) that they are irreversibly further metabolized, and are therefore not available for protein synthesis.

The first 2 possibilities were investigated in experiments described elsewhere (Geiger, '47). In those experiments it was not possible to detect any transitory storage of incomplete amino acid mixtures in the form of either amino acids or peptides. It was decided, therefore, to investigate by an indirect method whether administered amino acid mixtures, which by lack of 1 or more essential factors cannot be utilized for protein synthesis, are stored in some other way, so that after supplemental administration of the lacking amino acid protein synthesis can proceed. More specifically, it was planned to feed to young rats a diet lacking 1 or more essential amino acids during 12 hours, and then, in the next 12 hours to provide the missing amino acids by feeding a mixture containing besides carbohydrates, fats, vitamins, and salts, as the source of N *only* the amino acids which were missed from the preceding feeding.

It was assumed that if the incomplete mixture is stored in the body, protein synthesis and body growth will occur after the missing building stones have been provided. If, however, before feeding the supplement, the incomplete amino acid mixture was already in some way metabolized (deamination, gluconeogenesis, oxidation), then at the time of addition of

during the night and the other had diet 3 available during the daytime and diet 2 during the night.

The rats of group I, which received a complete diet (no. 1), served as controls. In group II, the rats had the chance to choose their food from the 2 jars; and on the basis of the experiments of Richter ('42-'43) concerning the behavior of rats in the self-selection of food, it was expected that the rats of this group would take from both jars such quantities that the food consumed *in toto* would support optimal growth. The rats of group III received the tryptophane-free amino acid mixture and the missing tryptophane at a 12-hour interval.

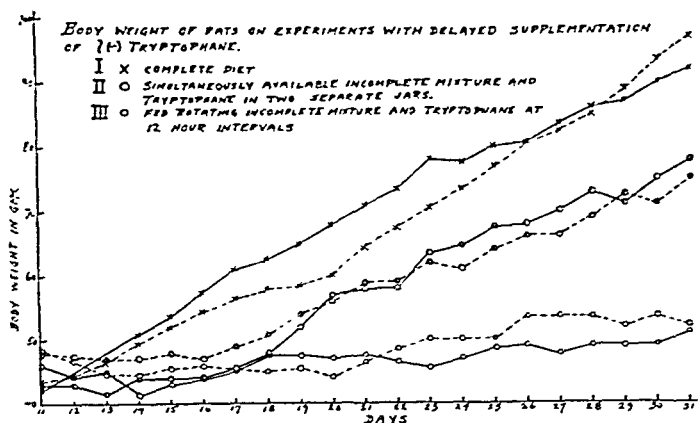


Figure 1

It was observed, however, that the rats did not consume the food immediately after it was put into the cages and therefore, the interval between intestinal absorption of the incomplete amino acid mixture and of the supplemented tryptophane varied considerably.

Figures 1 and 2 are self-explanatory. It is evident that the rats receiving the complete diet grew satisfactorily. The rats in group II which had the opportunity of supplementing diet 2 with diet 3, having both diets simultaneously present in different jars in the cage, did not grow for the first 6-7 days. In both experiments growth starts after this preliminary ad-

The whole system was always weighed together, so that small amounts of feed spilled into the outer jar were not lost. Besides these precautions, rats which did not have the habit of spilling their food were selected for these experiments.

TABLE 1  
*Composition of diets.*

COMPONENT	DIET NUMBER											
	1	2	3	4	5	6	7	8	9	10	11	12
Basal diet <sup>1</sup>	78.4	70	94	18.3	70	95	94	68	65	70	96	84.5
Acid hydrolyzed fish protein	19.5	30	..	...	..	..	..	..	..	..	..	...
Casein, oxidized	...	..	..	...	..	..	..	..	..	19	..	14
Zein	...	..	..	17.0	30	..	..	30	30	..	..	...
l(—)Tryptophane	2.1	..	6	0.5	..	5	..	2	..	1	..	0.5
l(+)Lysine	...	..	..	1.2	..	..	6	..	5	..	..	...
l(—)Cystine	...	..	..	...	..	..	..	..	..	1	..	0.5
dl-Methionine	...	..	..	...	..	..	..	..	..	..	4	0.5

<sup>1</sup> With the following composition: corn starch, 3050 gm (78.3%); rice bran concentrate, 400 gm (10.26%); cottonseed oil, 200 gm (5.13%); USP salt mixture, 200 gm (5.13%); fish oil (1 gm contains 2,000 I.U. vitamin A and 400 I.U. vitamin D) 50 gm (1.27%); riboflavin, 75 mg; Ca pantothenate, 150 mg; choline chloride, 2.5 gm.

## EXPERIMENTS

### *Experiments with tryptophane*

In the first group of experiments, tryptophane-free diets were fed, followed by a diet supplemented with the missing tryptophane. Rats of group I received the complete diet (no. 1) containing tryptophane and all other amino acids. The rats of group II had 2 jars in the cage, 1 containing diet 2 (lacking tryptophane) and the other containing diet 3 (containing the basal mixture plus tryptophane but lacking the acid hydrolyzed fish protein). Group III received for 12 hours diet 2 and in the next 12 hours diet 3. In order to rule out the effect of daylight on food intake, since rats are nocturnal animals, 1 of the rats received diet 2 during the day and diet 3

this amino acid does not promote growth when fed several hours after ingestion of a tryptophane-deficient diet. These results are in excellent agreement with the observation of Elman ('39) who found that when the injection of an amino acid solution which is deficient in tryptophane is followed after a 6-hour interval by the intravenous injection of tryptophane, positive N-balance cannot be achieved. Several authors have assumed that the utilization of parenterally administered tryptophane is different from that administered *per os* (for literature see Alcock, '36): therefore, the results obtained in our experiments with oral administration seem to be a significant confirmation of Elman's finding.

Somewhat similar experiments were published in 1929 by Berg and Rose. These authors showed that feeding large doses of tryptophane at long intervals (24-hour) is less effective than feeding smaller doses more frequently. Feeding half the daily allowance in the form of tablets at intervals of 12 hours induced better growth than when the total was administered at 1 time daily. The authors conclude that "apparently amino acids ingested in excess of anabolic needs are not retained."

Before drawing further conclusions from our results, we had to consider that according to the literature tryptophane is very rapidly eliminated from the body. Buck and Berg ('45) found that after oral administration of 0.35 to 1.0 gm tryptophane the tryptophane concentration in the blood increases rapidly, reaches its maximum after 2 hours and is again normal 5 hours after administration. The authors report also, that 1 hour after administration, only one-third of the fed tryptophane could be detected in the carcass. It was therefore assumed that tryptophane is very quickly destroyed either by deamination or by kynurenic acid formation.

Schweigert, Sauberlich, Elvehjem and Baumann ('46) also reported that after intraperitoneal administration of tryptophane, its concentration in the blood increases rapidly but returns to normal again in 2 hours.



justment period. A highly probable explanation for this observation is that after 5-6 days the rats learn to eat food rotating from the 2 jars at shorter intervals, so that an effective supplementation may be achieved. This possibility should be investigated in further experiments in which the time when the rats eat from the individual jars would be registered automatically. Finally, it is seen that there was no growth in group III in spite of the fact that the rats consumed considerable quantities of both diets 2 and 3.

BODY WEIGHT OF RATS ON EXPERIMENTS WITH  
DELAYED SUPPLEMENTATION OF 1% TRYPTOPHANE.

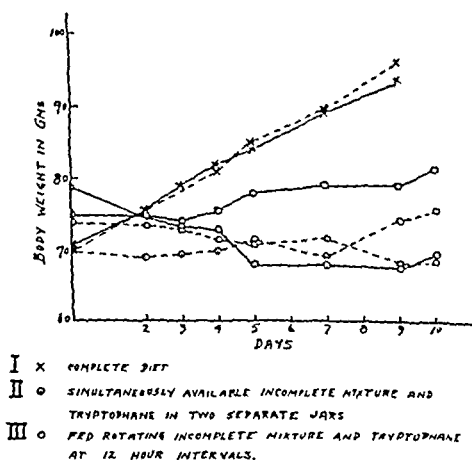


Figure 2

These experiments are interesting with respect to the ability of rats to select foods properly. Values of the ratio  $\frac{\text{ingested diet 2}}{\text{ingested diet 3}}$  were 2.3, 2.38, 1.78, and 2.5, which indicate that the rats selected an average of 1.0 gm of the tryptophane containing supplement for 2.24 gm of the tryptophane-deficient food. Figure 2 gives the results of a similar experiment of shorter duration.

The results of these experiments with tryptophane show that a delayed supplementation of tryptophane is ineffective;

weight in spite of the lysine supplementation, and this weight loss was more rapid than that observed by us earlier on rats fed diets entirely devoid of protein. It was assumed, therefore, that during the nitrite treatment, toxic substances were produced and that the presence of such substances rather than the destruction of lysine was responsible for the weight loss. This assumption was verified by experiments in which 10 gm of the treated casein were added to a regular diet containing 20 gm casein and 80 gm "basal diet." From experiments 7, 8 and 9 (table 2) it is evident that the control animal (no. 9) fed 20% casein and 80% "basal diet" gained as was to be expected, but the other animals (nos. 7 and 8) responded to the addition of nitrite-treated casein with loss of weight. The presence of toxic factors in casein treated according to Dunn and Lewis was assumed also by Hogan et al. ('41).

Another way to investigate the effect of delayed supplementation of lysine is to use zein as the source of lysine-free protein (Osborne-Mendel, '15; Rose and Rice, '39). This protein has to be supplemented with both tryptophane and lysine in order to secure growth in young rats and maintenance of weight in adult animals (Neuberger and Webster, '45; Gillespie, Neuberger and Webster, '45). The growth-promoting power of zein can be improved by adding to the diet these amino acids but even so, a normal rate of growth cannot be obtained. The reason for such failure has not yet been determined (Harris et al., '43; Borchers et al., '42; Kornberg, '46).

For the lysine-experiments (fig. 3), the rats of group I representing the control group, received diet 4 which contained both zein and the tryptophane and lysine supplements. The rats of this group grew sub-optimally as observed by Harris et al. ('43), and by Borchers et al. ('42).

Rats of group II had 3 jars in the cage simultaneously, 1 with diet 5 containing zein; 1 with diet 6 containing tryptophane, and the third with diet 7 containing lysine. It will be noticed that the rats of this group did not grow; in fact, they

In view of these data, it seemed possible that the results obtained in the present experiments with tryptophane were the result of the rapid destruction of this amino acid and may not represent a generally valid answer to the question whether incomplete amino acid mixtures are stored in the body. Therefore, it was decided to investigate whether the results observed in the experiments with tryptophane can be reproduced with other essential amino acids.

### *Experiments with lysine*

A lysine-free protein was readily prepared according to the procedure of Dunn and Lewis ('19). Casein was treated according to the method of these authors and fed as the sole

TABLE 2

*Experiments with casein treated according to Dunn and Lewis ('19).*

COMPOSITION OF DIET	RAT NO	BODY WEIGHT (GM) ON DAY OF FEEDING					
		Before	1	2	3	4	6
"Basal diet" 80 gm and treated casein 20 gm	1	42	40	35	31	29	27
	2	37	35	31	27	23	died
	3	106	104	86	72	65	60
"Basal diet" 80 gm, treated casein 20 gm, and lysine 1.5 gm	4	44	40	35	30	27	25
	5	40	40	34	28	died	
	6	120	116	102	88	77	63
"Basal diet" 70 gm, treated casein 10 gm, and casein 20 gm	7	115	108	95	82	77	75
	8	106	100	85	67	60	52
"Basal diet" 70 gm, casein 20 gm, and cellulflour 10 gm	9	115	121	126	130	137	145

protein source to rats. The data in table 2 show that rats fed such a diet lose weight very fast (see experiments 1, 2 and 3). It was assumed that lack of lysine was responsible for the weight loss and, therefore, the next group of rats was fed the same diet with the addition of 1.5 gm lysine to each 100 gm of the diet. These rats (experiments 4, 5 and 6) lost

### Experiments with methionine

A methionine-free casein was prepared according to Toennies ('42). The formic acid hydrogen-peroxide treatment used in this process destroys tryptophane as well as methionine. Therefore, it was necessary to supplement with tryptophane in order to carry out the experiment.

Three groups of rats were used. Control group I received the completed diet 12, and group II received simultaneously

BODY WEIGHT OF RATS ON EXPERIMENTS WITH  
DELAYED SUPPLEMENTATION OF METHIONINE

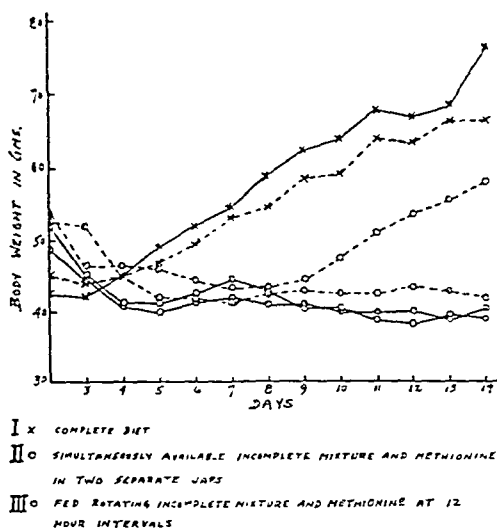


Figure 4

in 1 jar the methionine-free diet 10 and in the other jar diet 11 containing the methionine supplement. Group III received at 12-hour intervals diet 10 and diet 11 in rotation.

In this experiment, after the eighth day paired feeding for the rats of groups I and II was employed. It was determined how much food was consumed of diets 10 and 11 each by the rats of group II; corresponding quantities were then mixed and fed to the rats of group I next day.

From figure 4 it is evident that the fully supplemented oxidized casein (diet 12) has a fair growth-promoting property

lost weight. The animals did not show any sign of efficient self-selection of food (Scott and Quint, '46). Both preferred diet 7 containing lysine from which they ate relatively large quantities, but the amounts of diets 5 and 6 consumed were too small to permit growth.

The animals in group III were fed diet 8 containing zein and tryptophane for 12 hours, and diet 7 containing lysine for the next 12 hours.

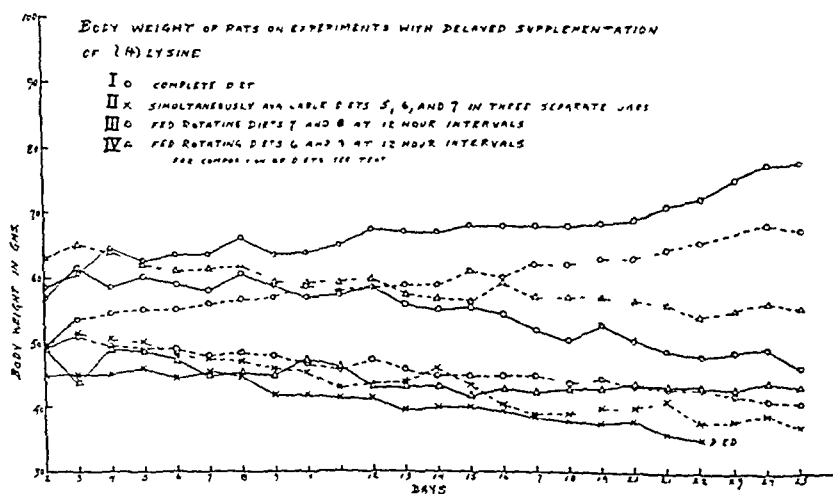


Figure 3

The rats of this group again conspicuously preferred diet 7 containing lysine but did not show any growth.

Finally, in group IV the animals were fed diet 9 containing zein and lysine for 12 hours, and diet 6 containing tryptophane, for the next 12 hours. In this group, sufficient quantities of both diets were consumed during 24 hours to produce growth if the food were properly utilized, but due to the delayed supplementation, the animals actually lost weight.

These experiments prove that the simultaneous presence of all the amino acids is essential not only in the case of tryptophane, but also with respect to lysine.

authors explain the different biological values of raw and processed soy protein by the observation, "that during digestion *in vivo* the methionine is released earlier from the heat processed soy meal than from the raw soy meal . . . in the case of raw meal, absorption occurs so late in the intestinal transit that this amino acid, as well as the incompletely supplemented amino acids, are not efficiently utilized for the synthesis of body protein."

Another bit of evidence in support of the assumption that for optimal protein synthesis the simultaneous presence of all the building stones is necessary, was supplied by Cox and Mueller ('44). These authors have shown that less essential amino acids are required for maintenance of N-balance, when the non-essential ones are also provided simultaneously. From their experiments it would seem highly probable that the non-essential amino acids, when not provided along with the essential ones, have to be synthesized in the body *ad hoc*. The synthesis of these building stones requires time during which some of the essential amino acids may be excreted or diverted from the protein synthesis by intermediary degradation or transformation.

#### SUMMARY

The fate of dietary amino acid mixtures which, because of absolute or relative lack of certain essential constituents, would either not be utilized or only partially utilized for protein formation was investigated.

With delayed supplementation of the lacking amino acid, it was shown that the missing tryptophane, methionine or lysine, when fed several hours after feeding of the "incomplete" mixture, does not promote growth. This finding supports the view that the "incomplete" amino acid mixtures are not stored in the body but are irreversibly further metabolized. It shows also that for protein synthesis all the essential components have to be present simultaneously.

as has already been found by Bennett and Toemmes ('42); the rats of group I were growing. However, the animals in groups II and III showed no growth.

One rat of group III, after losing weight for 10 days, suddenly started to regain its weight for no apparent reason.

These experiments with methionine supplementation prove that not only tryptophane and lysine, but also methionine, have to be present simultaneously with the other amino acids in order to provide favorable conditions for protein synthesis and whatever special metabolic processes these essential amino acids serve in the body.

#### DISCUSSION AND CONCLUSIONS

The experiments with delayed supplementation of lysine, methionine, and tryptophane are concordant in indicating that the essential amino acids have to be simultaneously present for protein synthesis. These findings show also that incomplete amino acid mixtures which by lack of an essential building stone cannot be used for protein synthesis, are not stored in the body but are further metabolized probably in an irreversible manner. These results should be considered when amino acid mixtures for therapeutic feeding are prepared or administered. The practical consequences of these findings will be further investigated.

This observation, that supplementing incomplete amino acid mixtures with the missing amino acid after a certain time interval does not promote growth, seems to constitute an important argument against the theory of step-by-step synthesis of protein molecules with intermediate formation of peptides. These results and those published by Berg and Rose ('29) and by Elman ('39), suggest that the formation of protein molecules is a coordinated tissue function and can be accomplished only when all amino acids which take part in the formation are present at the same time.

Two other papers may be cited in support of such a viewpoint (Melnick et al., '46; Russell et al., '46). The first named

# STABILITY OF REDUCED ASCORBIC ACID IN MARES' MILK<sup>1</sup>

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(Received for publication February 18, 1947)

In a previous study in which Holmes, Spelman, Smith and Kuzmeski ('47) compared the protein, mineral, and ascorbic acid content of mares' milk with that of the milk of other species, it was noted that the ascorbic value was relatively high. Since the mares' milk contained 4 or 5 times as much ascorbic acid as Holmes, Jones, Wertz, Esselen and McKey ('44) and Holmes Lindquist, Jones and Wertz ('45) obtained for milk from a herd of 5 common breeds of cows fed forage produced on the same or similar fields as that consumed by the mares, it was assumed that the difference was due to the 2 species of animals under consideration. This observation raised a question regarding factors that may influence the reduced ascorbic acid content of mares' milk, particularly since a sample of mares' milk that had been stored for several days, possessed approximately the same reduced ascorbic acid content as when it was freshly drawn from the mare, a condition quite different from the reported rapid disappearance of reduced ascorbic acid from cows' and human milk. Accordingly, a study was inaugurated to accumulate data regarding the stability of reduced ascorbic acid in mares' milk.

## EXPERIMENTAL

The milk was produced by mature Percheron mares at the end of the lactation period. Although the samples were col-

<sup>1</sup> Contribution No. 617 of the Massachusetts Agricultural Experiment Station.





The clear filtrate was divided into 10 ml aliquots, cooled in the refrigerator and titrated with the standardized dye solution. The bentonite served both to produce a clear filtrate and to greatly decrease the length of time required for filtering, for the proteins precipitated from mares' milk differ in appearance and physical condition from those of cows' milk. A series of duplicate tests gave the same results whether bentonite was used or omitted.

#### RESULTS AND DISCUSSION

The results of the individual assays of mares' milk for reduced ascorbic acid are reported in table 1. The range in initial values from 86 mg to 161 mg of ascorbic acid per liter makes it possible to compare the rate of disappearance of reduced ascorbic acid from mares' milk of widely different potencies. Samples VI, IX, XI, and XIII observed for a 10-day period, lost 2.6 mg, 2.3 mg, 2.5 mg, and 2.4 mg of reduced ascorbic acid per day, respectively; samples VII, X, XII, and XV, observed for 20 days, lost 2.5 mg, 1.9 mg, 1.5 mg, and 1.2 mg, respectively; samples III and V, observed for 28 days, lost 1.2 mg and 1.4 mg daily; samples I, II, IV, VIII, and XIV, during 33 days, lost 0.7 mg, 1.1 mg, 2.7 mg, 0.5 mg, and 2.1 mg, respectively. The rate of loss of reduced ascorbic acid from sample IV after 16 days' storage was radically different from that of the other samples. Omitting sample IV, the average daily loss of ascorbic acid from mares' milk stored for 10 days was 2.5 mg per liter, for 20 days was 1.8 mg, for 28 days was 1.3 mg and for 33 days was 1.1 mg. However, this correlation between the daily loss and the initial potency may be merely coincidental.

The 2.5 mg per liter daily decrease in reduced ascorbic acid during the first 10 days of storage agrees well with the 2.2 mg-per-liter loss reported by Gunsalus and Hand ('41) for fresh raw cows' milk. However, the decrease of 2.5 mg of ascorbic acid in mares' milk represents about 1.9% daily loss whereas the 2.2 mg decrease in cows' milk represents 14.8% loss. In other words, the loss of ascorbic acid from

lected during the late fall, pastures provided nearly all of the forage for the mares. However, since the season was unusually warm and sunny, the rainfall ample, and the pastures had been closely grazed, the grass that the mares consumed was young and green. The mares were in the pasture about 14 hours at night, and during the day they were confined in roomy box stalls where they received a supplementary ration of 4 quarts of cracked oats, a quart of whole corn and a small amount of early-cut, mixed hay. The milk samples, which were collected in the early morning, were protected from the light and taken directly to the laboratory, and the initial assay was started within an hour after the sample was obtained from the mare. The milk was stored in commercial flint glass milk bottles in a non-illuminated refrigerator at 10°C. When the milk was initially placed in storage, the bottles were completely filled and closed with the usual paper milk bottle cap. As aliquots were withdrawn for assay, the volume of air in the bottles constantly increased until when only the last aliquot remained the bottles were almost filled with air. This procedure seemed desirable since, in the average household, milk is frequently stored in the refrigerator in partially filled bottles.

When the study was begun, practically no information was available regarding the stability of the reduced ascorbic acid in mares' milk; therefore some of the samples were assayed daily. Other samples were assayed at longer intervals which served 2 purposes, i.e., materially increasing the period of observation and increasing the duration of the contact of air with the milk. The amount of reduced ascorbic acid in the 15 samples of mares' milk was determined by titration with sodium 2,6 dichlorobenzenonindophenol. A 25 ml sample of milk was measured into a 125 ml flask that contained a mixture of 5% phosphoric acid and 10% acetic acid. For clarification, 0.5 gm of bentonite<sup>2</sup> was added. The flask was stoppered and vigorously shaken for 1 minute and the mixture was filtered.

<sup>2</sup> U. S. Pharmacopocia, 12th revision, November 1, 1942, Mack Printing Co., Easton, Pa.

cows' milk is over 7 times as rapid as from mares' milk. In a study of 12 samples of commercial cows' milk stored at 1°C. for 6 days, Hand ('43) found a daily loss of 2.1 mg per liter or a loss of over 11%. Kothavalla and Gill ('43) observed a 24% loss from cows' milk stored at room temperature for 11 hours and a 26% loss during 3 days' storage at 5°C. Holmes and Jones ('45) found either only a trace or no detectable reduced ascorbic acid after a 30-minute exposure to sunshine. These observations are in accord with the data accumulated by several other investigators cited by Holmes and Jones ('45) which show that the reduced ascorbic acid content of cow's milk decreases rather rapidly during the first few days after the milk is obtained from the cow. Few data are available regarding the stability of ascorbic acid in human milk. Stateva ('43) reported that from 41% to 93% was lost during 24 hours' storage at room temperature.

While numerous investigators have supplied data regarding the effect of such factors as air, temperature, light, and oxidation upon the rate of disappearance of reduced ascorbic acid from cows' milk, a question naturally arises as to the primary factor which causes the destruction. Hand, Guthrie and Sharp ('38) reported that "lactoflavin is the sole agent in milk responsible for the sensitivity of ascorbic acid to light." In a study of the pigments, vitamins, and enzymes of milk in relation to changes in flavor and nutritive value, Hand and Sharp ('41) found that "riboflavin, the fluorescent green coloring matter in whey, is responsible for the oxidation of vitamin C in light." Thus one might assume that for a given set of conditions the more there is of naturally occurring riboflavin present in milk, the greater is the extent or the more rapid the disappearance of reduced ascorbic acid. This assumption is in a measure supported by the data under discussion, i.e., reduced ascorbic acid disappears more rapidly from cows' than from mares' milk and cows' milk contains more riboflavin than mares' milk. In one instance reduced ascorbic acid disappeared from cows' milk about 7 times as fast as from mares' milk; and Holmes, McKey, Wertz, Lind-

TABLE I  
*Stability of reduced ascorbic acid in mares' milk. All values are in mg/l.*

AGE OF MILK IN DAYS	SAMPLE														
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
0	86	94	99	104	111	116	119	121	123	124	131	147	149	157	161
1	..	..	..	..	..	114	120	..	122	123	130	146	148	157	159
2	..	..	..	104	..	112	119	..	118	122	130	145	145	156	158
3	..	..	93	..	..	109	..	..	116	..	127	..	139	..	..
4	81	92	..	..	104	106	..	..	112	..	125	..	139	..	..
5	..	..	..	100	..	..	111	121	105	117	..	140	139	153	..
6	79	84	..	..	..	..	108	..	..	113	..	138	132	..	154
7	..	..	..	93	..	..	102	..	..	..	..	132	..	..	150
8	..	74	..	..	96	..	..	..	..	..	..	..	..	..	..
9	..	..	78	88	..	93	98	..	..	..	..	..	..	140	..
10	..	..	..	..	..	90	..	..	..	..	107	129	..	..	..
12	71	..	..	..	..	..	..	100	..	..	106	..	125	119	..
13	70	..	..	..	..	..	..	..	..	..	..	..	..	..	..
14	..	66	..	..	..	..	..	..	..	..	..	..	..	..	..
15	..	..	..	..	..	..	..	..	99	99	..	..	..	..	..
16	..	..	..	..	..	82	117	..	99	..	..	..	..	140	..
20	..	..	85	76	..	..	117	..	..	..	..	117	..	140	..
28	..	73	..	85	..	..	117	..	..	..	..	..	..	..	..
33	..	66	..	73	..	68	..	..	87	..	..	..	110	..	..
62	57	..	16	..	..	..	109	..	..	..	..	117	..	136	..
Total loss	24	37	33	88	38	26	51	16	23	37	25	30	24	69	25
Av. loss/dn.	0.7	1.1	1.2	2.7	1.4	2.6	2.5	0.5	2.3	1.9	2.5	1.5	2.4	2.1	1.2

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quist and Parkinson ('46) found cows' milk contained about 10 times as much riboflavin as mares' milk produced on the same farm. Rasmussen ('46) stated that cows' milk contains approximately 10 times as much riboflavin as mares' milk, and Rasmussen, Bogart and Maynard ('38) observed that sunlight did not decrease the ascorbic acid content of mares' milk to the extent that it did for cows' milk. However, it is quite evident that the available data are too meager to permit any definite conclusions regarding any relationship between the amount of riboflavin present in milk and the rate of disappearance of ascorbic acid.

#### SUMMARY

Fifteen samples of mares' milk with initial potencies of from 86 mg to 161 mg of reduced ascorbic acid per liter were stored in the dark at 10°C. They were assayed at daily or longer intervals. Four samples observed for 10 days lost an average of 2.5 mg per liter daily; 4 samples stored 20 days lost 1.8 mg per day; 2 stored 28 days lost 1.3 mg daily; and 3 observed for 33 days lost 1.1 mg per liter per day. These data show that the rate of loss of reduced ascorbic acid from mares' milk is only a fraction of the rate of loss from cows' milk.

#### ACKNOWLEDGMENT

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# IS COBALT A DIETARY ESSENTIAL FOR THE RABBIT? <sup>1</sup>

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Of the endemic diseases caused by a deficiency of the micro-nutrient elements, that due to a lack of cobalt is perhaps the most widespread. In this country alone, localities in Michigan, New Hampshire, and Wisconsin have recently been added to the already long list of cobalt deficient areas (Baltzer et al., '41; Keener et al., '44; Geyer et al., '45). A great deal of work has been done in attempting to clarify the role that this element plays in animal physiology — for recent reviews see Marston ('39), Schultze ('40), Underwood ('40), and Maynard ('41) — yet much remains to be learned. While the decreased and depraved appetite, progressive debility, anorexia, and general emaciation are characteristics of cobalt deficiency in sheep and cattle, none of these symptoms is specific.

Attempts to produce a cobalt deficiency in the rabbit and guinea pig in the hope of finding specific symptoms or changes were unsuccessful. The work is reported briefly since it indicates that there is a species difference in the need for cobalt.

## EXPERIMENTAL

The basal diet consisted of whole milk<sup>3</sup> and corn grain.<sup>4</sup> The average of several determinations for the cobalt content

<sup>1</sup> Taken from a Ph.D. thesis by the senior author. Cornell University, 1944.

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<sup>3</sup> Obtained from the Cornell University herd, milked into Pyrex jars.

<sup>4</sup> Grown in a low-cobalt area, obtained through the courtesy of Dr. G. K. Davis, Florida Agricultural Experiment Station, Gainesville, Florida.





diet, the animals were sacrificed and the livers taken for cobalt analysis.

### RESULTS AND DISCUSSION

At 4 weeks of age the hemoglobin values were below normal, ranging between 5 and 9.9 gm/100 ml of blood, because until this age the animals had received only milk. The hemoglobin values for the animals receiving the complete mineral supplement, as well as for those of the animals receiving the supplement lacking only cobalt, rose to normal in about 2 weeks after starting the supplement, while the values for the 2 groups receiving a supplement of manganese alone rose more slowly. After 8 weeks, at which time the hemoglobin levels of the latter 2 groups were still subnormal, 1 of these was given a supplement of iron and the other of copper. This was done to determine if possible whether the anemia found was due to the lack of copper or of iron.

The hemoglobin levels rose slightly, the animals of the group receiving iron responding a little more quickly than those receiving copper; however, the difference in response was small and probably of no significance. At 14 weeks of age the iron and copper supplement was given to all groups; from this point on, then, the 15 surviving animals can be considered as falling into 2 groups, 1 group of 5 receiving cobalt and the other of 10 receiving no cobalt. The groups were not divided equally because it was hoped that if cobalt deficiency symptoms should develop, there would be an opportunity to subdivide the larger group and supplement the diet of part of them with cobalt. The hemoglobin values from this point to the end of the experiment were 12.9 for the group receiving cobalt and 12.6 gm/100 ml for those not receiving cobalt. These values are not significantly different and are somewhat below the value of 14.0 gm/100 ml characteristic of our colony on a stock diet.

At 6 months of age, the 4 surviving females were bred. Three had young, none living for more than a few days. Later attempts to obtain viable young led to similar results.

of the milk was 0.0007  $\mu\text{g}/\text{ml}$ , while the corn contained 0.0005  $\mu\text{g}/\text{gm}$  dry weight. In the proportions eaten by the animals, this made a diet containing 0.0024 p.p.m. on a dry basis. These analyses and those on liver samples were made by the method of Ellis and Thompson ('45).

Preliminary experiments showed that good growth could be obtained with the rabbit fed this diet, but that with the guinea pig it is unsatisfactory. Sprouting the grain to a length of 6 to 8 inches made a noticeable but insufficient improvement. In this preliminary work it became evident that the requirement for cobalt by both of these species was not high.

A second experiment was performed using rabbits. Twenty animals were distributed at random into 4 groups. From birth to 4 weeks of age they were denied access to the dam's feed, but were allowed to suckle at intervals and were given fluid whole milk and whole milk powder. At 4 weeks of age they were placed in individual glass cages. In addition to the basal diet fed ad libitum, 1 group was given orally twice weekly a supplement of iron, copper, manganese, and cobalt at a rate to give, respectively, 2,000, 200, 2,000 and 4  $\mu\text{g}$  per animal per day. Another group was treated in similar fashion except that the cobalt was omitted. The remaining 2 groups were supplemented only with manganese, since it was expected on the basis of the iron and copper content of the diet that a mild nutritional anemia might be produced and that under these conditions a cobalt deficiency might be more easily demonstrated.

The manganese was purified by the extraction of a solution of manganous chloride with o-nitrosocresol in petroleum ether, while the iron was purified by extraction from strong HCl with diethyl ether according to the method of Dodson et al. ('36). Baker's c.p. cupric sulfate and cobaltous sulfate were used as the source of copper and cobalt.

The animals were weighed weekly and their condition noted. Hemoglobin determinations were made periodically by the method of Sanford and Sheard ('30). After 46 weeks on the

surviving until the end of the experiment, the average cobalt content of the livers of the cobalt-supplemented rabbits was  $0.140 \mu\text{g/gm}$  while the cobalt content of the other animals was  $0.014 \mu\text{g/gm}$  of dry tissue. The livers of those animals which died during the course of the experiment contained  $0.14 \mu\text{g/gm}$  for the cobalt-supplemented group and  $0.029 \mu\text{g}$  cobalt  $\text{gm}$  of dry tissue for the controls.

At autopsy the animals were carefully examined and for the most part appeared normal in the gross with the exception that the livers of nearly all of the animals of both groups were cirrhotic. This finding as well as the mortality rate and the failure of these animals to reproduce makes it clear that the diet used is not complete for these species. It may be that a deficiency of some other dietary essential was partially responsible for the failure to produce a cobalt deficiency.

Wunsch ('39), having reviewed the studies in New Zealand, states that forage from 77 pastures where trouble occurred contained on an average  $0.038 \text{ p.p.m.}$  cobalt on a dry matter basis, and Underwood and Harvey ('38) reported a comparable figure of  $0.04 \text{ p.p.m.}$  based on Australian studies. The present diet containing  $0.0024 \text{ p.p.m.}$  on a dry basis then gives an extremely low cobalt intake compared to that required by the sheep or cow and suggests that there is a species difference in the requirement of various animals for cobalt. Additional support for the idea that the rabbit requires less cobalt than the sheep or cow lies in the fact that the dried liver contained only  $0.014 \text{ p.p.m.}$  of cobalt as compared with the values of  $0.06 \text{ p.p.m.}$  and  $0.02 \text{ p.p.m.}$  in affected sheep reported by Underwood and Harvey ('38) and Askew and Dixon ('37), respectively.

Marston ('39) in a review article mentions a personal communication from McDonald to the effect that rabbits show no signs of cobalt deficiency when fed entirely on pasture from affected areas, and according to Marston the same is true for the horse. Working with the rat, Underwood and Evehjem ('38) found the requirement to be less than  $0.6 \mu\text{g}$ , and Houk et al. ('46),  $0.03 \mu\text{g}$  per animal per day.

Although growth was fairly good (see table 1) the mortality rate during the experiment was high, there being 10 survivors at the end of the 46 weeks. Various attempts to reduce the mortality rate by supplemental feeding of sodium chloride, thiamine, and cellulose seemed to be of no help. Although growth was slightly more rapid and the mortality rate slightly lower in the cobalt-supplemented group, the difference was not significant.

TABLE 1

*Average weight and number of survivors at monthly intervals.*

MONTHS ON EXPERIMENT	COBALT SUPPLEMENT		NO COBALT SUPPLEMENT	
	Wt. gm	No.	Wt. gm	No.
0	336	5	343	15
1	618	5	589	14
2	893	5	712	12
3	1260	5	1053	10
4	1495	5	1242	8
5	1560	5	1384	8
6	1649	4	1400	7
7	1719	4	1435	7
8	1835	4	1753	7
9	1866	4	1728	7
10	1944	3	1848	7
10.5	1906	3	1768	7

A record of the feed consumption was kept for a period of 8 months starting from the beginning of the experiment. Four of the animals fed the cobalt supplement and 7 of those not receiving cobalt survived this period. In the former group the average consumption of corn by each rabbit per day was 34 gm, while the corresponding figure for the latter group was 29.5 gm. The corresponding values for milk consumption were 140 gm and 129 gm per animal per day. Thus, the slightly better growth response of the group receiving cobalt corresponds with a slightly greater feed consumption, but here again the difference is not significant. During this period of 8 months, the average daily intake of cobalt of the non-supplemented group was 0.1  $\mu$ g as compared with 4  $\mu$ g fed as a supplement to the other group. Considering only those animals

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There is thus a growing body of evidence that cobalt deficiency is limited to the ruminants and more particularly to sheep and cattle. Some years ago Filmer ('33) suggested that some organic factor is lacking in cobalt deficiency, on the basis of their work showing that liver is effective in curing "Coast Disease," the effectiveness not being accounted for by the liver ash.

More recently McCance and Widdowson ('44) cite a personal communication from C. J. Martin that sheep suffering from "Coast Disease," while cured by cobalt given by mouth, are not cured by cobalt when given by injection. They suggest further that the element probably acts upon some of the organisms in the rumen. We suggest, further, that the microorganisms affected are those concerned with the synthesis of B-vitamins. Many of the symptoms of cobalt deficiency are comparable to those found in B-vitamin deficiency. Anorexia, for example, is a symptom of both cobalt and thiamine deficiency. Hypochromic microcytic anemia and impaired reproduction are common symptoms in both cobalt and pyridoxine deficiency.

#### SUMMARY

Rabbits fed whole milk and corn grain require less than 0.1  $\mu$ g of cobalt per animal per day. This finding is discussed in the light of the hypothesis that the need for cobalt is peculiar to ruminant species and may be concerned primarily with biological processes in the rumen.

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The Mead Johnson and Company Award for Research on the Vitamin B Complex was presented to *Dr. William J. Darby*, Associate Professor of Biochemistry, Vanderbilt University School of Medicine, *Dr. Paul L. Day*, Professor of Physiological Chemistry, University of Arkansas School of Medicine, and *Dr. E. L. R. Stokstad*, Research Chemist, Lederle Laboratories, for their discoveries and investigations in the field of the B-complex vitamins with special reference to their studies on the chemical nature and nutritional significance of folic acid. The presentation was made at the annual dinner of the American Institute of Nutrition on Monday evening, May 19, in Chicago.





# THE IMPORTANCE OF RIBOFLAVIN, PANTOTHENIC ACID, NIACIN AND PYRIDOXINE IN THE NUTRITION OF FOXES <sup>1</sup>

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TWO FIGURES

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The nutritional requirements of the fox have been under investigation in our laboratory in which purified casein-sucrose rations supplemented with synthetic vitamins have been used. The requirements for folic acid and another unidentified factor or factors in fresh liver have been reported (Schaefer et al., '47). Since fox pups receiving the purified ration supplemented with all the known synthetic vitamins grow as well as fox pups receiving a stock meat, cereal, and liver ration for a period of 14-16 weeks, it is possible to study the requirement for these vitamins if the deficiencies develop within 12 to 14 weeks after being placed on the deficient ration. Experiments were initiated, therefore, in which each of the following vitamins was omitted from the complete basal ration: riboflavin, pantothenic acid, niacin or pyridoxine.

## EXPERIMENTAL

Weanling silver and red fox pups 6 to 7 weeks of age were used in these studies. The general experimental procedure has been described previously (Schaefer et al., '47). The

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The Borden Award for 1947, administered by the American Institute of Nutrition, was presented to *Dr. L. A. Maynard* for many years of noteworthy services in the field of general nutrition and for many significant contributions to the knowledge of the physiology of milk secretion, of factors affecting milk production and milk composition, and of the requirements of animals for dietary fats. The presentation was made at the annual dinner of the American Institute of Nutrition on Monday, May 19, in Chicago.

of growth of all the pups showed a definite decrease. Pup 19 was fed a single supplement of 2 mg riboflavin and normal growth resumed. However, 3 weeks later failure was extremely sudden, and the animal died before another supplement could be given. After 3 to 4 weeks on the riboflavin deficient ration, pups 14 and 13 exhibited acute deficiency

TABLE 1  
*Ration = Basal — Pyridoxine.*

PUP. NO.	DAYS ON EXP'T.	WEIGHT	HEMOGLOBIN	HEMATOCRIT	R.B.C.
		<i>kg</i>	<i>gm %</i>	<i>%</i>	<i>per cm<sup>3</sup></i>
24	1	1.60	8.99	26.9	
	31	1.86	9.26	22.0	7.17
	49	2.22	6.86	17.0	4.35
	49	Fed 3 mg pyridoxine orally and started 0.2 mg pyridoxine per 100 gm ration			
	63	2.62	10.90	34.0	9.0
25	1	1.55	7.35	30.8	
	31	1.78	8.21	17.5	5.67
	49	2.09	5.60	12.5	4.22
	52	died			
28	1	2.96	14.16	42.0	10.11
	69	3.11	14.67	43.8	10.33
	101	3.20	10.89	37.0	10.22
		Started 0.20 mg pyridoxine per 100 gm ration			
	121	3.33	15.48	54.2	10.94
Av. of	49	2.89	11.80	35	8.70
4 pups on	70	3.56	12.49	40.0	9.24
complete	98	4.07	14.63	42.4	9.60
basal					

symptoms characterized by muscular weakness followed by clonic spasms and coma. Pup 13 was fed 1 dose of 5 mg riboflavin orally and pup 14 received 5 mg orally and 1.25 mg intravenously. Within 2 hours the animals regained muscular control and consciousness. A normal gain in body weight followed. Three weeks later growth in both animals was retarded, opacity of the lens and diminution of pigment production in the underfur and guard hair was noted. Therapy

basal ration had the following composition: sucrose 66%, casein (Smaco Vit. Test) 19%, cottonseed oil 8%, cod liver oil 3% and salts IV (Phillips and Hart, '35) 4%. Each 100 gm of ration was supplemented with 0.2 mg thiamine chloride, 0.2 mg pyridoxine hydrochloride, 0.4 mg riboflavin, 1.5 mg calcium pantothenate, 4 mg niacin, 100 mg choline chloride,

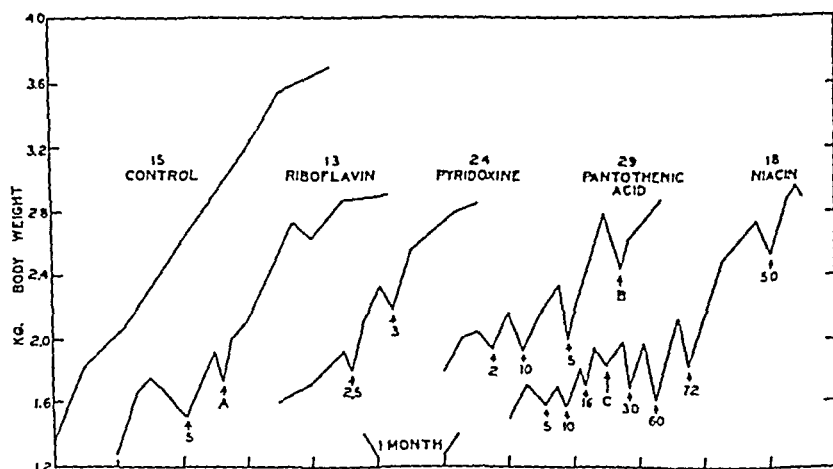


Fig. 1 Growth of fox pups demonstrating the effect of omitting 1 vitamin from the basal ration. Numbers (i.e., 5) equal mg of therapy of the specific vitamin omitted. Fox 15 — complete basal. No. 13 — basal ration minus riboflavin. A, started 0.125 mg per 100 gm ration. No. 24 — basal ration minus pyridoxine. No. 29 — basal ration minus pantothenic acid. B, 20 mg calcium pantothenate orally plus 5 mg intravenously in a solution of glucose and saline. No. 18 — started on the basal ration minus niacin and folic acid. C, 30 mg niacin orally and started 0.1 mg folic acid per 100 gm of ration.

0.1 mg folic acid, 0.025 mg biotin, 25 mg i-inositol, 50 mg p-aminobenzoic acid, 2 mg alpha-tocopherol and 0.5 mg 2-methyl-1,4-naphthoquinone. Growth curves of a typical fox pup from each series of experiments are given in figure 1. Table 1 summarizes the blood findings.

### *Riboflavin*

Red fox pups nos. 13, 14 and silver pup 19 received the basal ration minus riboflavin. After 2 weeks on experiment the rate

*Pantothenic acid*

Five pups, 4 red and 1 silver, were placed on the basal ration minus calcium pantothenate. After 2 to 3 weeks on experiment growth of the pups ceased although food consumption remained normal. Failure was extremely sudden in pups 11 and 12 and death resulted when the animals had been on experiment for 26 and 27 days, respectively. When pup 30 had been on experiment for 18 days and a definite loss in body weight was evident, the ration was supplemented with 0.25 mg calcium pantothenate per 100 gm ration. The gain in body weight was rapid for 8 weeks; however, failure was sudden and the animal died before additional therapy could be given.

Pup 16 responded once to calcium pantothenate when in a mild state of deficiency. Later the more severe form was allowed to develop, which was characterized by a deep coma. Intravenous injection of a solution of calcium pantothenate, glucose and saline resulted in relief from the coma in 1 hour; however, the pup was found dead the following morning. Pup 29, as shown in figure 1, after responding 3 times to calcium pantothenate therapy was allowed to develop a severe deficiency which was characterized by clonic spasms and this condition was followed by coma. A solution of calcium pantothenate, glucose and saline was injected intravenously, and 30 minutes after therapy the pup had recovered from the coma and was running about the cage. A significant increase in body weight followed.

Necropsies performed on all pups immediately after death revealed fatty degeneration of the liver, catarrhal gastroenteritis, and cloudy swelling and congestion of the kidneys. The deficiency symptoms, the dramatic response to pantothenic acid, and the results of macroscopic pathological examination of pups which died of the deficiency were similar to those observed in dogs (Schaefer et al., '42a). The preliminary results obtained with pup 30 indicates that the requirement for pantothenic acid is greater than 0.25 mg per 100 gm ration.

consisting of 0.125 mg and 0.250 mg riboflavin per 100 gm of ration for pups 13 and 14, respectively, was initiated when the first signs of muscular weakness were noted. Both animals gained weight rapidly; however, after a period of 9 weeks following the daily supplementation of the ration with ribo-



Fig. 2 Pelts of wild red fox pups 13 and 14 which developed a riboflavin deficiency and then received 0.125 mg and 0.250 mg riboflavin, respectively, per 100 gm of ration. Pelt on the right is that of a red fox pup receiving the complete basal ration supplemented with liver. (Note the white underfur on pelts 13 and 14).

flavin, pup 14, which received 0.25 mg, weighed 740 gm more than pup 13.

The lack of proper pigmentation of the fur continued, and 6 to 7 weeks after riboflavin therapy was initiated the underfur and guard hair were white. Pigmentation of the guard hair gradually returned and was approaching normal, whereas the underfur was still white when the pups were pelted (fig. 2). The symptoms, such as loss in weight, muscular weakness, coma and opacity of the lens were similar to those observed in riboflavin deficient dogs (Potter et al., '42).

*Pyridoxine*

Silver pups 24 and 25 and red pup 28 were placed on the basal ration minus pyridoxine. Five ml blood samples were collected from the radial vein at selected intervals. Hemoglobin determinations were done according to the method of Evelyn ('36); hematocrit readings were obtained with Wintrobe tubes. Blood analyses are given in table 1. Anorexia and arrest in growth occurred in pups 24 and 25 after being on experiment for 5 weeks. 2.5 mg pyridoxine were administered orally and the animals gained weight for 3 weeks. At this time the animals again developed anorexia, appeared listless and were anemic. Hemoglobin values were 6.86 and 5.6 gm % for pups 24 and 25, respectively, as compared to 11.8 gm % for animals receiving the complete basal ration. Pup 25 was in a comatose state when pyridoxine therapy was started and failed to recover. The oral feeding of 3 mg pyridoxine and supplementation of the ration thereafter with 0.2 mg per 100 gm to pup 24 resulted in a rapid response in growth and alleviation of the anemia. The animal was killed accidentally before it could be determined whether complete remission of the anemia would result from continued pyridoxine therapy.

Pup 28 was approximately 20 weeks of age when placed on experiment. The body weight curve showed a plateau and after 17 weeks on experiment the hemoglobin level dropped from 14.67 to 10.89 gm %. At this time the ration was supplemented with 0.20 mg pyridoxine per 100 gm ration. Hemoglobin was increased to 15.48 gm %; however, no significant increase in body weight was noted.

## DISCUSSION

From these experiments it is evident that fox pups require riboflavin, pantothenic acid, niacin and pyridoxine. The deficiency symptoms observed when 1 of the above vitamins is omitted from the ration closely resemble those reported in similar studies with dogs (Potter et al., '42; Schaefer et al., '42a, b; McKibbin et al., '42). The rapidity with which the



### *Niacin*

Silver pups nos. 17, 18 and 23 received the basal ration minus niacin and folic acid. Within 2 weeks anorexia was apparent and the pups failed to gain weight. The administration of 5 to 30 mg niacin resulted in resumption of growth. During the first 5 weeks on experiment pup 18 (fig. 1) developed 3 deficiencies with subsequent responses to niacin. The ration was then supplemented with 0.1 mg folic acid per 100 gm ration and 4 more deficiencies, with subsequent response to single dosages of varying levels of niacin, were obtained.

After 3 deficiency and treatment periods, during the first 6 weeks on experiment, the ration for pup 17 was supplemented with folic acid. One week later loss in body weight was evident and the oral administration of 26 mg niacin failed to alleviate the deficiency. The subsequent feeding of 70 gm fresh liver produced a rapid gain in body weight.

Pup 23 during the first 4 weeks on experiment developed 2 deficiencies with subsequent responses to niacin. The ration was then supplemented with folic acid. After 2 additional deficiencies and responses to niacin the animal lost weight rapidly. The oral administration of 86 mg niacin, 2 mg folic acid, 10 times the daily supplement of the remainder of the B-complex and the intramuscular injection of 20 mg nicotinamide, failed to correct the deficiency and the animal died. The failure of the animals to respond to niacin after repeated deficiencies, and the rapid response to fresh liver is very similar to the results obtained with dogs given the same treatment (Ruegamer et al., '47).

Typical blacktongue symptoms noted in dogs (Schaefer et al., '42b) such as loss in weight, anorexia, inflammation of the gums, palatine redness, and diarrhea were noted in the foxes where a severe deficiency was allowed to develop. The requirement for nicotinic acid as calculated by single dose feedings for growing pups varied greatly among assays with the same pup and among pups (range of 0.39 to 2.0 mg per kg body wt. per day).

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deficiencies develop, especially of riboflavin, pantothenic acid or niacin, is very striking and emphasizes the importance of insuring adequate quantities of these vitamins in rations for growing fox pups.

Although our experiments were not designed to study the exact requirements of fox pups for the above vitamins, the following minimum and maximum levels may be suggested for future work: Riboflavin — greater than 0.125 mg and less than 0.4 mg per 100 gm of ration (0.2 mg for the dog). Pantothenic acid — greater than 0.25 mg and less than 1.5 mg per 100 gm of ration (0.20 mg for the dog). Niacin — 0.39 mg to 2.0 mg per kg of body weight per day (0.25 mg to 0.36 mg for the dog) as calculated from single dose feeding. Pyridoxine — less than 0.20 mg per 100 gm of ration (0.12 mg for the dog).

The changes occurring in the pigmentation of the fur of the foxes on the riboflavin experiment are of extreme interest. This diminution of red color may have been due to riboflavin and in part or entirely to the lack of unknown factors supplied by liver, since a depigmentation of the underfur has been observed in silver fox pups receiving the complete basal ration (Schaefer et al., '47). The quality of the fur was not affected.

#### SUMMARY

The importance of riboflavin, pantothenic acid, niacin and pyridoxine in the rations for growing fox pups has been clearly established. The deficiency symptoms when 1 of the above vitamins is missing from the ration closely resemble those observed in dogs. The rate with which the deficiencies develop is as rapid, and in numerous instances even more rapid, in fox pups than in dogs.

#### ACKNOWLEDGMENTS

We wish to acknowledge our indebtedness to Merck and Company, Rahway, N. J., for the crystalline vitamins; and to Lederle Laboratories, Inc., Pearl River, N. Y., for the synthetic folic acid.

# DIET OF MOTHER AND BRAIN HEMORRHAGES IN INFANT RATS<sup>1</sup>

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TWO FIGURES

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Several investigators have shown that young rats grow to maturity at a rapid rate and bear litters when fed a synthetic ration containing vitamins A and D, alpha tocopherol, thiamine, riboflavin, pyridoxine, pantothenic acid, niacin and choline. The diets in some cases (Richardson, Long and Hogan, '42; Richardson and Hogan, '46; Climenko and McChesney, '42; Ershoff, '46) contained 2-methyl-1,4-naphthoquinone. In other cases (Jukes, '40; Unna, '40; Henderson et al., '42; Vinson and Cerecedo, '44) this compound was not added to the diet but the vitamin probably was supplied by some constituent such as corn oil or cottonseed oil, or by bacterial synthesis in the digestive tract of the mother.

Synthesis of vitamin K in the intestinal tract of the rat has been demonstrated by several investigators. Greaves ('39) has reported a decrease in blood prothrombin and a prolonged bleeding time in rats with bile fistulas. Oral administration of large amounts of vitamin K concentrates or smaller amounts combined with bile salts restored clotting time to normal. Black et al. ('42), Day et al. ('43), and Kornberg et al. ('44) observed a high incidence of severe hypoprothrombinemia in

<sup>1</sup> Authorized for publication as Technical Article no. 1039 of the Texas Agricultural Experiment Station.



a period of several years. Vitamins A and D were supplied by percomorph oil.<sup>2</sup> Lard and vitamin K were the only variable constituents of the experimental diets. The amounts of these constituents in the different diets and the observations on the young of the females which received the diets are summarized in table 2. The females in group 1 received the basal diet, diet A, which contains both lard and vitamin K. Those in group 2 received diet B which contains vitamin K but no lard. Those in group 3 received diet C which contains lard but no vitamin K, and those in group 4 received diet D which contains neither lard nor vitamin K.

TABLE 2  
*Relation of the diet of the mother to brain hemorrhages in infant rats.*

GROUP NO.	DIET NO.	VARIABLE CONSTITUENTS		YOUNG				
		Lard	Vitamin K <sup>1</sup>	Born	Hemorrhagic brains		Weaned	
		%	mg/100 gm	No.	No.	%	No.	%
1	A	10	2.5	167	0	0	89	53.2
2	B	..	2.5	142	0	0	49	34.5
3	C	10	..	63	0	0	31	49.2
4	D	..	..	157	82	52.2	18	11.4

<sup>1</sup> 2-Methyl-1,4-Naphthoquinone.

Female rats were given either diet A or diet C at 28 days of age and continued on the same diet until they weighed 175 to 200 gm. At this time they were either continued on the same diet or transferred to another experimental diet and mated with a male from the stock colony. The females which were transferred to diet B had been reared on diet A and those which were transferred to diet D had been reared on diet C.

*Diet of mother and brain hemorrhages.* When the diet contained both lard and vitamin K (group 1) the offspring were normal and 89 out of 167 (53.2%) were weaned. Likewise the offspring were normal when the diet of the mother contained either vitamin K (group 2) or lard (group 3) separately,

<sup>2</sup> Mead, Johnson and Company.

rats receiving diets that contained sulfonamides to prevent sufficient intestinal synthesis of vitamin K. When the vitamin K was fed with sulfonamide the hypoprothrombinemia did not develop. These data indicate that vitamin K acts in rats as it does in other animals to maintain a normal prothrombin level in the blood. The Syrian hamster requires (Hamilton and Hogan, '44) vitamin K for a normal rate of growth and presumably for normal reproduction. Moore et al. ('42) found a low prothrombin level in the blood of pregnant rabbits that had received diets deficient in vitamin K. These females aborted at the end of the first trimester of gestation.

Some investigations have been carried out in this laboratory during the past year on the relation of the diet of the mother to abnormalities in the offspring. A high incidence of brain hemorrhage has occurred in the offspring of mothers which received a synthetic diet deficient in vitamin K and low in fat. Some of the observations on this abnormality are described in this report.

#### EXPERIMENTAL

The composition of the basal diet of the mothers is given in table 1. This diet contains all the recognized vitamins with the exception of vitamin C and pteroylglutamic acid, and it has been reasonably satisfactory in reproduction studies over

TABLE 1  
*Composition of basal diet.*

	<i>gm</i>	<i>Vitamins per 100 gm</i>	
Casein	25	Vitamin A	3000 I.U.
Cerelose	57	Vitamin D	425 I.U.
Woodpulp	3	Alpha tocopherol <sup>1</sup>	2.5 mg
Salt mixture <sup>2</sup>	5	Menadione <sup>1</sup>	2.5 mg
Lard	10	Thiamine <sup>1</sup>	1.0 mg
Choline <sup>1</sup>	0.1	Riboflavin <sup>1</sup>	1.0 mg
Inositol <sup>1</sup>	0.01	Pyridoxine <sup>1</sup>	1.0 mg
p-Aminobenzoic acid <sup>1</sup>	0.05	Calcium pantothenate <sup>1</sup>	4.0 mg
		Niacin <sup>1</sup>	5.0 mg
		Biotin <sup>1</sup>	0.02 mg

<sup>1</sup> Supplied by Merek and Co., Rahway, N. J.

<sup>2</sup> Richardson and Hogan ('46).

total of 58 young were born in the first litters. Severe hemorrhages occurred in 20 (34.5%), and 14 young (24.1%) were weaned. The same females produced 61 young in subsequent litters and brain hemorrhages occurred in 45 (73.7%) and none were weaned. Although brain hemorrhages constituted the most characteristic abnormality, hemorrhages occurred also in the body cavity in a few young.

*Whole blood coagulation time.* The coagulation time of blood of 12 mothers which produced hemorrhagic young and of 6 young with visible hemorrhages in the brain was determined by Duke's capillary tube method. It ranged from 60 to 120 seconds in both the mothers and the young and is considered normal since it did not differ from that of rats in the stock colony.

Since both lard and vitamin K in the diet of the mother protect the young from hemorrhages, it seemed desirable to test the vitamin K activity of lard with chicks. Day old New Hampshire Red chicks were used in these tests and the experimental period was 2 weeks. The basal diet was similar to that described by Richardson, Hogan and Karrasch ('42). Pteroylglutamic acid was supplied by 10% of ether-extracted dried brewers' yeast. The average coagulation times of blood of chicks which received diets containing different amounts of lard with and without vitamin K for 2 weeks are summarized in table 4. When the diet contained vitamin K (groups

TABLE 4

*Average coagulation time of blood of chicks receiving diets that contain different amounts of lard with and without vitamin K.*

GROUP NO.	LARD %	VITAMIN K mg/100 gm	COAGULATION TIME <sup>1</sup>	
			No. of chicks	Min.
1 C	10	2.5	5	3.3
2 C	0	2.5	5	3.8
3 C	0	0	4	30 plus
4 C	5	0	4	30 plus
5 C	10	0	4	22
6 C	20	0	4	24

<sup>1</sup> Experimental period — 2 weeks.



but a higher percentage of the young was weaned when the diet contained lard without vitamin K than was weaned when it contained vitamin K without lard. There was a high incidence of brain hemorrhages in the offspring of females (group 4) which received neither lard nor vitamin K. Brain hemorrhages occurred in 82 out of 157 (52.2%) of the young and only 18 (11.4%) were weaned.

*Incidence of brain hemorrhages.* The observations on the young which are given in group 4 of table 2 have been rearranged in table 3 to show the incidence of brain hemorrhages in the first litters as compared to that in subsequent litters.

TABLE 3

*Incidence of brain hemorrhages in first as compared to subsequent litters.*

GROUP NO	DIET NO.	NO OF FEMALES	LITTER SEQUENCE	BORN	YOUNG HEMORRHAGIC BRAINS		WEANED	
				No.	No.	%	No.	%
4a	D	6	1	38	17	44.7	4	10.5
4b	D	12	1	58	20	34.5	14	24.1
			2 to 6	61	45	73.7	0	0
4ac <sup>1</sup>	C	6	2 to 4	29	0	0	17	58.6

<sup>1</sup> Same females as described in 4a. After a female had borne 1 litter, she was transferred to diet C which contained lard but no vitamin K.

The 6 females in group 4a were each allowed to produce 1 litter on diet D. A total of 38 young were born in the first litters and 17 (44.7%) developed severe brain hemorrhages. Only 4 (10.5%) were weaned. The females (group 4ac) were then transferred to diet C and allowed to bear additional litters until it was evident that no more young would be born. A total of 29 young were born in subsequent litters and none were hemorrhagic. Seventeen out of the 29 (58.6%) were weaned. The addition of lard to the diet decreased the incidence of brain hemorrhages to zero and increased the per cent of young weaned.

The 12 females in group 4b received diet D from the time they were mated until the observations were discontinued. A

total of 58 young were born in the first litters. Severe hemorrhages occurred in 20 (34.5%), and 14 young (24.1%) were weaned. The same females produced 61 young in subsequent litters and brain hemorrhages occurred in 45 (73.7%) and none were weaned. Although brain hemorrhages constituted the most characteristic abnormality, hemorrhages occurred also in the body cavity in a few young.

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Since both lard and vitamin K in the diet of the mother protect the young from hemorrhages, it seemed desirable to test the vitamin K activity of lard with chicks. Day old New Hampshire Red chicks were used in these tests and the experimental period was 2 weeks. The basal diet was similar to that described by Richardson, Hogan and Karrasch ('42). Pteroylglutamic acid was supplied by 10% of ether-extracted dried brewers' yeast. The average coagulation times of blood of chicks which received diets containing different amounts of lard with and without vitamin K for 2 weeks are summarized in table 4. When the diet contained vitamin K (groups

TABLE 4

*Average coagulation time of blood of chicks receiving diets that contain different amounts of lard with and without vitamin K.*

GROUP NO.	LARD	VITAMIN K	COAGULATION TIME <sup>1</sup>	
			No. of chicks	Min.
	%	mg/100 gm		
1 C	10	2.5	5	3.3
2 C	0	2.5	5	3.8
3 C	0	0	4	30 plus
4 C	5	0	4	30 plus
5 C	10	0	4	22
6 C	20	0	4	24

<sup>1</sup> Experimental period — 2 weeks.

1C and 2C) the whole blood coagulation time was normal regardless of whether or not the diet contained lard. When the diet did not contain vitamin K (groups 3C, 4C, 5C and 6C) the whole blood coagulation time was prolonged regardless of the amount of lard present. However, it was longer in the groups which received no lard (group 3C) and only 5% of lard (group 4C) than in groups 5C and 6C which received 10 and 20% of lard, respectively. These tests show that lard, when fed to chicks, supplies a small but insignificant amount of vitamin K. This small amount might be sufficient when fed to rats at a level of 10% to meet the requirement for reproduction under normal conditions. However, we do not believe that this explanation is entirely adequate, in view of the fact that the whole blood coagulation time of hemorrhagic rats is normal. If the reports by Black et al. ('42), Day et al. ('43), and Kornberg et al. ('44) that a prolonged coagulation time and a hypoprothrombinemia are typical of a vitamin K deficiency are correct, these data suggest that brain hemorrhages are not due entirely to a deficiency of this vitamin. A possible explanation of this discrepancy would be that some substance is produced in the body under normal conditions which maintains normal capillary fragility. This substance is not synthesized when the diet is low in fat and deficient in vitamin K at the same time.

*Age at death.* The age at which the infant rats died with brain hemorrhage is shown in table 5. Most of the young which died with brain hemorrhages were either born dead or died within 24 hours after birth. The hemorrhages were

TABLE 5  
*Age at death of rats with hemorrhagic brains.*

<i>Age in days</i>	<i>No. of rats</i>
Birth	22
1	45
2 to 5	3
8 to 11	5
15 to 18	4
20 to 21	3

visible in a few young while they were still living, but death always occurred within a few hours when the hemorrhages became visible in living young that were less than 5 days old. The larger rats usually became unstable 2 to 3 days before death and in a few cases a dark area was visible underneath the skull. Every rat which developed these symptoms was autopsied at death and brain hemorrhages had occurred in every case. Hemorrhage occurred after the fifth day only in first litters.

In a second test brain hemorrhages developed in only 10 out of 72 young which were born to 8 females. The incidence of brain hemorrhages was about the same in the first as it was in the second litters. So far we are unable to account for the low incidence of hemorrhages in this series.

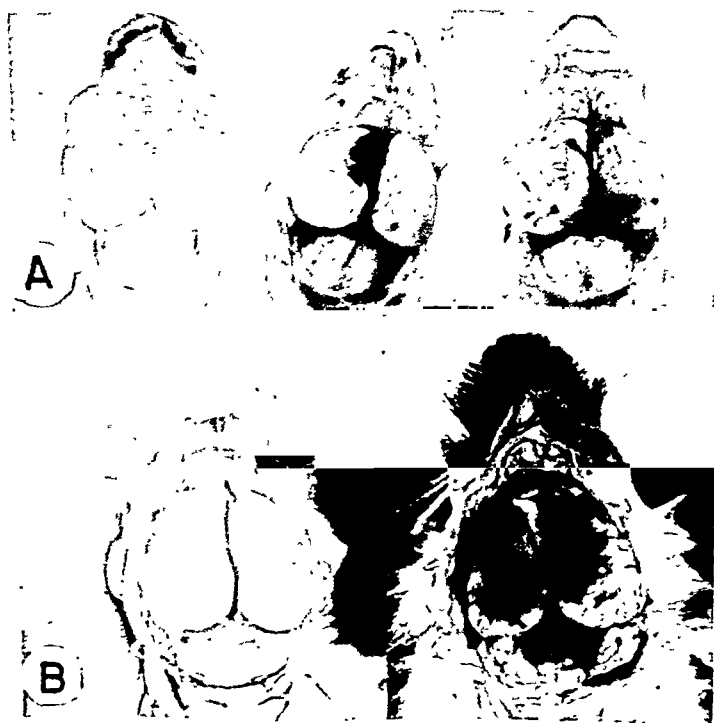


Fig. 1 Normal and hemorrhagic brains. (A) Day-old rats. (B) Twenty-one-day-old rats.

Photographs of the brains of a normal and of hemorrhagic rats 1 day and 21 days old are shown in figure 1. Microphotographs<sup>3</sup> of sections of a normal and of a hemorrhagic brain are shown in figure 2. Large areas of blood in the tissues are visible in the hemorrhagic brain. In the preparation of the slides for the microphotographs the tissue was fixed and

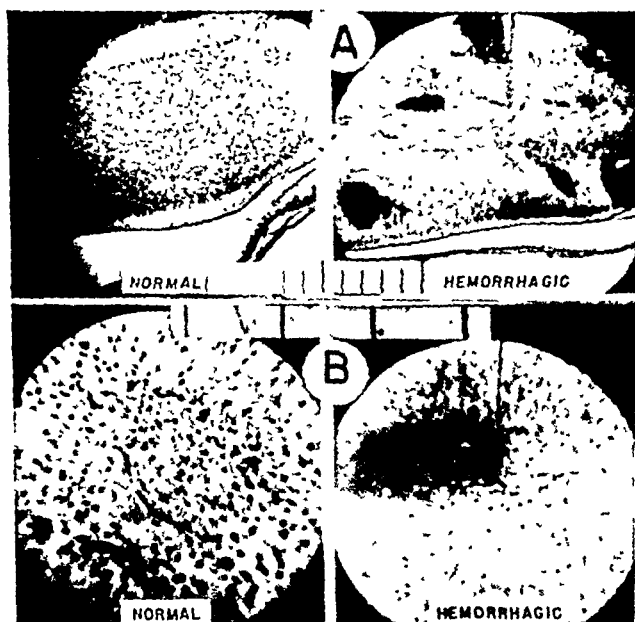


Fig. 2 Sagittal section of brain through cerebrum of a normal and of a hemorrhagic rat. A, low power; B, high power. One division of stage micrometer scale equal to 0.1 mm.

decalcified in Bouin's fixative and stained by Lillie's modification of Masson's trichrome stain.

*Reports of brain hemorrhages by other workers.* Brain hemorrhages in infant rats have been reported previously by 2 groups of workers. Moore et al. ('27) reported brain hemorrhages in a few of the young born to females which

<sup>3</sup> We are indebted to Dr. Sidney O. Brown, Associate Professor of Biology, for the preparation of sections and slides and for the microphotographs.

had been reared and allowed to bear young on a synthetic diet that contained 2% of dried yeast as the only source of the water-soluble vitamins. The diet contained 3% of hydrogenated cottonseed oil<sup>4</sup> as a source of fat. The hemorrhages were thought to be due to a low level of vitamin B because they did not occur when the yeast was increased to 7%. Jervis ('42) reported the occurrence of brain hemorrhages in infant rats from mothers who were fed a synthetic diet deficient in choline. Three females received the experimental diet which contained 10% hydrogenated cottonseed oil and each female received a daily supplement of 100 µg each of thiamine, pyridoxine, riboflavin and calcium pantothenate and 6 mg of niacin. The 2 control females received essentially the same diet with 100 mg of choline added to each 100 gm of the diet. The young from the mothers on the choline deficient diet ceased to grow at the tenth to twelfth day and 50% of them died with severe nervous symptoms. Extensive hemorrhagic lesions of the cerebellum were the most characteristic abnormality found on histological examination of the nervous system of 5 young which exhibited the very severe nervous symptoms. Vitamin K was not included in the diet of the females described in either of these reports and both investigations used hydrogenated cottonseed oil<sup>4</sup> as the source of fat. We are investigating the effect of this particular fat on the production of brain hemorrhages at the present time, but it seems very probable that the hemorrhages described in the previous reports, as well as those described here, are due to the same deficiency.

#### SUMMARY

Female rats were reared to maturity on a synthetic diet that was deficient in vitamin K. If lard was removed from their diet and the females allowed to bear litters, a high incidence of brain hemorrhages occurred in the offspring. The hemorrhages did not occur when the diet contained either lard or vitamin K.

<sup>4</sup> Crisco.

The incidence of hemorrhage in first litters was 34.5 to 44.17% and in subsequent litters, 73.7%.

Most of the young that died with brain hemorrhages were either born dead or died within 24 hours after birth. A few young in the first litters developed hemorrhages between the eleventh and twenty-first days.

The whole blood coagulation time of females which produced hemorrhagic young and of young with visible hemorrhages was normal. This shows that the prothrombin level was not abnormally low and it is suggested that some substance which acts normally to maintain capillary strength is not synthesized by the body when the diet is low in fat and deficient in vitamin K. An assay with chicks shows that lard may contain a small but insignificant amount of vitamin K.

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# AVAILABILITY OF CAROTENE FROM CARROTS AND FURTHER OBSERVATIONS ON HUMAN REQUIREMENTS FOR VITAMIN A AND CAROTENE<sup>1</sup>

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Carrots occupy a unique place among the vegetables as a potential contributor to the vitamin A value of the *human* diet in the United States not only because they have by far the highest carotene content of any food commonly eaten by man (U. S. Bureau of Human Nutrition and Home Economics, '45), but also because they are consumed in large quantities (U. S. Bureau of Human Nutrition and Home Economics and National Research Council, '44). However, there is evidence that the ability of the human body to absorb and utilize the large amounts of carotene present in carrots is very limited (Eckelen and Pannevis, '38; Kreula and Virtanen, '39; Lister Institute and Medical Research Council, Vitamin A Sub-committee of the Accessory Food Factors Committee, '45).

Employing the dark adaptation reaction as a criterion of the vitamin A status of the individual, Booher and Callison ('39) and Booher, Callison and Hewston ('39) in this laboratory have established a procedure for measuring the degree of utilization of vitamin A by man and have determined the

<sup>1</sup> This research was supported by an allotment made by the Secretary of Agriculture from Special Research Funds (Bankhead-Jones Act of June 29, 1935).

tion of phloem to cortex was present in the piece as in the whole slice. The remainder of the slice was preserved for chemical analysis by storing at  $-40^{\circ}\text{C}$ . The bioassays were conducted essentially according to the procedure outlined in the U. S. Pharmacopoeia (Twelfth ed.), 108 animals being used. Crystalline  $\beta$ -carotene<sup>2</sup> in cottonseed oil was employed as the standard, since it has been found that the U.S.P. reference cod liver oil no. 2 is unsuitable for use as a standard of reference (Callison and Orent-Keiles,<sup>3</sup> 45).

### *Chemical analysis*

The pooled aliquots of carrots, 1 cooked and 1 uncooked for each subject, were extracted according to the method of Moore and Ely ('41). The extracts were chromatographed on a 1:2 mixture of magnesium oxide<sup>3</sup> and Hyflo Supercel. The  $\alpha$ - and  $\beta$ -carotene fractions were eluted separately with purified petroleum ether, fraction F, and absorption of each fraction was determined at 450 m $\mu$ , using a Coleman spectrophotometer, Model 10-S. The amount of  $\alpha$ - or  $\beta$ -carotene present in its respective fraction was calculated from a standard  $\beta$ -carotene curve obtained on the same instrument. The standard  $\beta$ -carotene curve was used in estimating both  $\alpha$ - and  $\beta$ -carotene, since Zscheile, White, Beadle and Roach ('42) have shown that the absorption coefficients at 450 m $\mu$  are practically identical for these 2 isomers. The bands at the top of the magnesia column, which amounted to only 4% of the total carotenoids, were not investigated further.

### RESULTS AND DISCUSSION

The time required for the first depletion of these subjects ranged from 2 to 6 months, reflecting the great individual variation in vitamin A reserves that had been observed in previous experiments in this laboratory. The second deple-

<sup>2</sup> S.M.A.  $\beta$ -carotene.

<sup>3</sup> E. S. Good reagent, tested in this laboratory for ability to give good separation and recovery of  $\alpha$ - and  $\beta$ -carotene.

tion period was much shorter and more uniform. In every case the scotopic visual threshold began to rise as soon as the carrot supplement was discontinued and within 13 to 16 days had reached a level 0.5 to 0.7 log units above the subject's normal average threshold. At this time feeding of the second carrot supplement was begun. Both periods of depletion were in progress during the spring and summer months, so that the seasonal elevation of visual threshold associated with winter, which has been reported by English investigators (Lister Institute and Medical Research Council, Vitamin A Sub-committee of the Accessory Food Factors Committee, '45) was not encountered.

TABLE 2

*Vitamin A potency of carotene-containing supplements as measured by chemical and bioassay methods.*

SUPPLEMENT	VITAMIN A VALUE MEASURED BY		DIFFERENCE BETWEEN PREDICTED AND BIOASSAY VALUE <sup>2</sup>
	Chemical Analysis	Bioassay	
	I.U./gm	I.U./gm	%
Crystalline carotene in cottonseed oil <sup>1</sup>	$1.44 \times 10^6$	$1.67 \times 10^6$	+ 15.9
Carrots, frozen, cooked <sup>2</sup>	146	50.5	- 65.4
Peas, frozen, cooked <sup>1</sup>	6.2	7.0	+ 12.9
Spinach, frozen, cooked <sup>1</sup>	85	97.5	+ 14.7

<sup>1</sup> U.S.P. reference cod liver oil no. 1 used as standard in bioassay.

<sup>2</sup> S.M.A.  $\beta$ -carotene used as standard in bioassay.

Cooking, over and above the amount required for blanching the carrots preparatory to freezing, caused no differences in either the carotene content of the vegetable or the ability of the human to utilize the carotene. The same amount of carrots whether cooked or uncooked was required by each subject for restoration of normal vision.

There was a marked discrepancy between the vitamin A value of the carrots calculated from the chemical analysis, using  $0.6 \mu\text{g}$   $\beta$ -carotene and  $1.2 \mu\text{g}$   $\alpha$ -carotene, respectively, as equivalent to 1 I.U. of vitamin A, and that same value measured directly by the rat-growth bioassay procedure (table 2).

Chemical analyses of the samples were in close agreement and gave an average of 77.8  $\mu\text{g}$   $\beta$ -carotene and 19.4  $\mu\text{g}$   $\alpha$ -carotene or a calculated total of 146 I.U. vitamin A value per gm of carrots. The vitamin A activity of duplicate samples of carrots determined by rat-growth bioassay was 50.5 I.U. per gm, equivalent to only one-third of the provitamin A shown to be present in the vegetable by chemical analysis.

It is obvious (table 3) that there can be a three-fold difference in the statement of the vitamin A requirement of these human subjects, depending upon which value, chemical or biological, is used in calculating the potency of the carrots from which practically the entire vitamin A intake of the

TABLE 3

*Spectrophotometric vs. biological method of assay of carrots in estimating the vitamin A requirement of human adults.*

VITAMIN A REQUIREMENT <sup>1</sup> BASED ON:	S U B J E C T							
	6		7		8		9	
	VIT A IN I U. PER		VIT A IN I U. PER		VIT A IN I U. PER		VIT. A IN I U. PER	
	day	kg/day	day	kg/day	day	kg/day	day	kg/day
Spectrophotometric analysis	6645	115	8110	101	5185	90	4450	85
Bioassay	2345	40	2860	36	1770	31	1590	30

<sup>1</sup> Figures are inclusive of small amount of vitamin A present in basal diet.

subjects was derived. Table 4 compares the results from this investigation with findings obtained previously in this laboratory (Booher and Callison, '39; Booher, Callison and Hewston, '39). In these earlier studies a similar method of experimentation was used, the dietary sources of vitamin A being U.S.P. reference cod liver oil no. 1, crystalline carotene in oil, cooked spinach or cooked peas. In these studies also (table 2) chromatographic as well as biological assays were made of the provitamin A active constituents of all the carotene-containing supplements, U.S.P. reference cod liver oil no. 1 with a rated potency of 3000 I.U. per gm being used as the standard of reference.

It has been demonstrated by Callison and Orent-Keiles ('45) and by Porter, Nash, Zscheile and Quackenbush ('46)

TABLE 4  
Summary of apparent vitamin A requirements of humans as affected by method of measurement of vitamin A  
value from different sources.

APPARENT HUMAN REQUIREMENTS FOR VITAMIN A DERIVED FROM (All values expressed in international units per kg body weight)						
Subject	Reference cod liver oil, no. 1; <i>Assay by</i> <i>Hio.</i>	Crystalline Carotene in oil; <i>Assay by</i> <i>Chem.</i> <i>Bio.</i> <sup>1</sup>	Carrots frozen uncooked; <i>Assay by</i> <i>Chem.</i> <i>Bio.</i> <sup>2</sup>	Carrots frozen cooked; <i>Assay by</i> <i>Chem.</i> <i>Bio.</i> <sup>2</sup>	Peas frozen cooked; <i>Assay by</i> <i>Chem.</i> <i>Bio.</i> <sup>1</sup>	Spinach frozen cooked; <i>Assay by</i> <i>Chem.</i> <i>Bio.</i> <sup>1</sup>
1	36	50 57				
2	56	88 103				
3	52	78 90				
4	25	37 42				
5	34	52 60				
6			115 40	115 40		
7			101 36	101 36		
8			90 31	90 31		
9			85 30	85 30		
10		66 76			42 47	
11		83 96			51 57	68 77
12		92 106				76 87
13						88 101
Average	40.6	68.2 78.8	97.8 34.2	97.8 34.2	46.5 52.0	77.3 88.3

<sup>1</sup> U.S.P. reference cod liver oil no. 1 used as standard in assay.

<sup>2</sup> S.M.A.  $\beta$ -carotene used as standard in assay.

that U.S.P. reference<sup>1</sup> oils nos. 2 and 3, respectively, are unstable and undependable as standards in vitamin A assays. Although there are less actual direct experimental data to be cited in proof, the stability of U.S.P. reference cod liver oil no. 1 has also been questioned by Morton ('42) and most recently by the United States Bureau of Dairy Industry's Technical Committee in charge of the nation-wide survey ('47) to determine the value of butter as a source of vitamin A in the diet of the people of the United States. Wiseman<sup>4</sup> analyzed immediately upon delivery, a sample of U.S.P. reference cod liver oil no. 1 which had been in transit less than 24 hours and was packed in dry ice during that time. After removal of an aliquot for analysis, the bottle was resealed under nitrogen, stored at reduced temperature and reopened at weekly intervals for a period of 3 weeks, the same precautions being observed at each subsequent resealing and storage. The absorption of the non-saponifiable fraction at 325 m $\mu$  was measured by means of a photoelectric spectrophotometer, using a Hilger double monochromator. The values for  $E_{1\text{ cm}}^{1\%}$  325 m $\mu$  were 1.50, 1.42, 1.38 and 1.38, respectively. These figures indicate a fall of 8% in the vitamin A potency of the oil during a 3-week period under storage conditions which should keep such losses at a minimum.

In view of these observations on the instability of U.S.P. reference cod liver oil no 1, it is important to reevaluate the bioassay values reported by Booher and Callison ('39) for the crystalline carotene, cooked spinach and cooked peas, in which the standard was U.S.P. reference oil no. 1, and to compare them with the carotene content of these supplements as measured chemically. The bioassay indicated a vitamin A potency for crystalline carotene 15.9% higher than would have been predicted from the carotene analysis, and values 12.9 and 14.7% higher than calculated for the cooked peas and cooked spinach, respectively, assuming complete utilization of carotene from the vegetables (table 2). An uncorrected decrease in the actual potency of the standard of reference employed in a

<sup>4</sup> Wiseman, H. G. Unpublished data.

bioassay leads to just such an apparent increase in the vitamin A value of the material being assayed as reported by Callison and Orent-Keiles ('45). It is, of course, impossible to determine whether or not the entire amount of the discrepancy between the 2 values can be accounted for in this way. Since no spectrophotometric measurements were made of the samples of reference cod liver oil no. 1 used in the assays, the extent of the deterioration of the standard is unknown. However, Callison and Orent-Keiles ('45) have demonstrated that errors of from 30 to 44% can actually occur in vitamin A assays, due to deterioration of U.S.P. reference cod liver oil.

Since U.S.P. reference cod liver oil no. 1 was used in establishing the human requirement for preformed vitamin A by Booher, Callison and Hewston ('39), these values are also probably somewhat too high. On the other hand, the quantitative relationship between the human requirement for preformed vitamin A and for carotene established in this study remains unchanged, since any correction necessitated by the instability of the U.S.P. reference oil no. 1 applies equally to both the cod liver oil and carotene preparations used as supplements. This relationship appears to be remarkably constant for the 5 subjects studied. On the basis of international units per kilogram, subject no. 1 required 62% as much preformed vitamin A as of vitamin A value provided by carotene in oil. Subjects nos. 2, 3, 4, and 5 required 54, 58, 58, and 57% respectively. Since in every case the human "requirement" in terms of the bioassay vitamin A value of the carotene, is greater than the "requirement" in terms of preformed vitamin A, it is apparent that the human being experiences somewhat greater difficulty than the rat in utilizing carotene in this form.

As pointed out above, deterioration in the reference oil may account for the higher values of vitamin A potency obtained by bioassay as compared with chemical analysis of carotene in oil, peas, and spinach. These differences ranged from at least 13 to 16% in magnitude. On the other hand, the lower value obtained for carrots must definitely indicate the



rat's inability to utilize carotene from carrots as effectively as from the other sources. Crystalline  $\beta$ -carotene was the standard used in the vitamin A bioassay of carrots. Therefore the question of instability of reference oil does not enter into consideration of the bioassay figures for this vegetable.

Comparison of the human vitamin A "requirements" calculated from the chemical analyses of the several supplements (table 4) demonstrates that carotene from different sources varies in its availability to the *human* organism. For subjects 10, 11 and 12, carotene from a food source appears to be more fully utilized than carotene in oil. Subjects 10 and 11 required only 63 and 61% as much carotene, respectively, when derived from peas as from crystalline carotene fed in oil. Subjects 11 and 12 required 81 and 83% as much carotene, respectively, when derived from spinach as pure carotene in oil. Unfortunately, it was impossible to study the ability of subjects 6, 7, 8 and 9 to utilize carotene in oil, since the experiments were forced to terminate because the subjects were called to military service.

The rat was able to use carotene from carrots only about one-third as well as from peas, spinach or pure carotene in oil. It is possible that the *human being* utilizes carotene from carrots more efficiently than does the rat. On the basis of the bioassay values, the "requirement" of all 4 subjects for carotene from carrots is below the range found for the 9 subjects receiving carotene from the other sources studied, in all but 1 case (subject 4 for pure carotene). This is true even when the bioassay figures are decreased by 13 to 16% to adjust for possible deterioration of the reference cod liver oil.

In this connection it may be noted, however, that the amount of carotene utilized from carrots by the *human* subjects in this study to support normal dark adaptation is consistent with the recent observation of the Vitamin A Sub-committee of the Accessory Food Factors Committee, Lister Institute and Medical Research Council ('45) that 75% of the carotene

from dietary carrots was eliminated in the feces, leaving 25% available to the body. No details of the chemical methods employed were given in the preliminary report, however.

Inasmuch as neither chemical analyses of carotene content nor bioassays of vitamin A potency are necessarily indicative of the actual availability of this nutrient to the human being, clear-cut differentiation should be made between (1) the vitamin A or carotene content of a food as determined by chemical analysis (2) the vitamin A or carotene requirement of the animal, including man, as determined by the amount of crystalline carotene or vitamin A preparation necessary for the support of a specific physiological function (for example growth or normal dark adaptation), and (3) the efficiency of utilization of the nutrient from a given food to meet these requirements. Graves ('42), Bacharach ('41) and Pyke ('42) have discussed some of the difficulties encountered in interpreting data for practical use and suggestions for overcoming these difficulties have been offered. However, any general factors suggested at this time to be applied as human availability corrections to the vitamin A or carotene values appearing in dietary tables are purely empirical and vary with different foods.

The results of this study emphasize the need for caution when making use of carotene and vitamin A values found in current food composition tables for the planning of diets and evaluation of their adequacy with respect to *human* requirements for this vitamin. In addition, they point to the need for further investigation of vitamin A and carotene utilization by man by means of balance or digestion studies paralleled by similar experiments with rats, since the latter are the standard animals employed for the vitamin A bioassay. It is important to include a large number of human individuals in such a study, since variability in this species is extremely great. Such studies, though difficult and time-consuming, will yield information of value in bringing order out of the confusion which now exists in this field.

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# THE ARGININE REQUIREMENT OF YOUNG TURKEY POULTS

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## ONE FIGURE

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It has been shown that chicks require arginine for maintenance and growth (Arnold et al., '36; Klose et al., '38; Klose and Almquist, '40). Rats, on the other hand, can synthesize arginine at a rate which permits maintenance but which is insufficient for optimum growth (Burroughs et al., '40). The need for arginine by the turkey poult has not been investigated previously. Three experiments were completed in which young poults were fed an arginine-low ration supplemented with various levels of arginine to determine whether poults require this amino acid, and if they do, the amount necessary for optimum growth.

## EXPERIMENTAL

The basal ration used in experiments 1 and 2 contained the following per 100 gm: water-washed casein, 26.6 gm; l(-) cystine, 0.4 gm; glycine, 2.0 gm; calcium gluconate, 5.0 gm; fish oil (3000 A-400 D), 0.5 gm; synthetic vitamins, 0.20591 gm; soybean oil, 3.0 gm; special salt mixture, 1 gm<sup>1</sup>; cholic acid, 0.1 gm; hexane extract of alfalfa, equivalent to 2.0 gm alfalfa; folic acid equivalent to 4 gm solubilized liver; and glucose to make 100 gm. The special salt mixture contained (in %) 96.47

<sup>1</sup> Composition: tricalcium phosphate, 3.5 gm; dipotassium phosphate, 1.29 gm; potassium chloride, 0.3 gm; magnesium sulfate, 0.4 gm; sodium silicate, 0.2 gm.

sodium chloride, 0.49 manganese, 0.1 iron, 0.05 copper, 0.05 zinc, 0.05 aluminum, 0.002 cobalt and 0.04 iodine. The synthetic vitamins included choline chloride 0.2 gm, biotin 0.00001 gm, alpha tocopherol 0.001 gm, thiamine 0.0005 gm, riboflavin 0.0005 gm, pyridoxine 0.0004 gm, d-pantothenic acid 0.002 gm, and nicotinic acid 0.0015 gm. For the third experiment the basal ration was essentially the same as in the previous tests, but the casein was increased to 28% and pteroylglutamic acid<sup>2</sup> was substituted for the folic acid concentrate. The l(+) arginine monohydrochloride, l(-)cystine, and glycine used were commercial preparations.

Bronze turkey poults were used in these experiments. At hatching they were placed in electrically heated batteries with raised wire floors and fed a regular starter mash. At 1 week of age, the poults were divided into equivalent weight groups of 9 poults each in the first 2 experiments and 8 each in the third test. The experimental diets and water were supplied *ad libitum*. The first and second experiments were continued for 10 days each and the third experiment for 12 days.

## RESULTS AND DISCUSSION

Block and Bolling ('45) have reported that casein contains 4.1% arginine on a 16% nitrogen basis. Since the washed casein in the present series of experiments contained 82.5% crude protein, it was assumed that it contained 3.4% arginine. This value was used throughout in calculating the arginine content of the rations.

Data for the 3 experiments are shown in figure 1. The percentage gains per day were calculated according to the following formula:

$$\text{percent gain per day} = \frac{\text{average gain} \times 100}{\text{average body weight} \times \text{number of days}}$$

The results agree well with those to be expected except for the groups in experiments 1 and 2 which were fed 1.15%

<sup>2</sup>The pteroylglutamic acid was kindly supplied by Lederle Laboratories, Inc., Pearl River, N. Y., through the courtesy of Dr. T. H. Jukes.

arginine: these gained less than the groups fed slightly less (1.07%) arginine. In each of the first 2 experiments the highest level of dietary arginine produced the greatest gain, with a greater gain on the 1.31% than on the 1.23% level (fig. 1). These results indicated that the optimum level had not been exceeded but since the gains made by the poult fed the highest level of arginine were only slightly less than the maximum gains expected on this type of diet, the optimum level could not be greatly in excess of 1.31%. To determine this optimum level, the amount of arginine was increased to

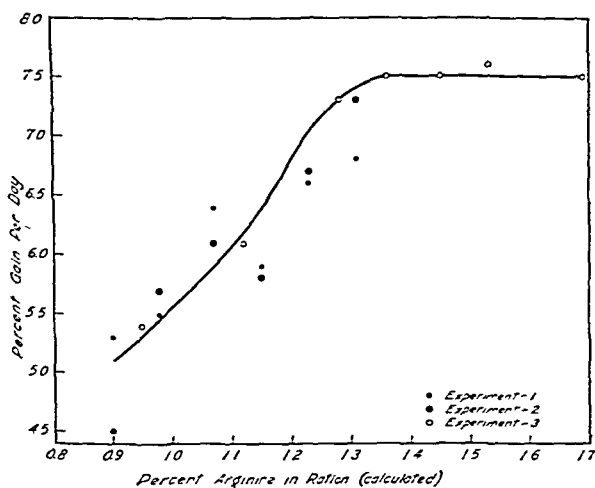


Fig.1 The relation of gain in weight of young poult to the level of arginine in their ration.

1.69% of the ration in the third experiment. The growth curve showed a plateau at a level of approximately 1.35% of arginine.

Mortality did not seem to be related to the level of arginine fed, with the possible exception of the first test in which 2 poult died in the group receiving the lowest level of arginine, while 1 poult died in the group receiving 1.07% arginine. In the second test 2 poult died in each of the groups receiving 0.98 and 1.31% arginine and 1 poult in each of the groups receiving 0.90, 1.07 and 1.23% arginine. In the third trial,



the only poult that died was in the group receiving 1.12% arginine.

The poult requirement of approximately 1.35% arginine, as indicated in figure 1, is considerably greater than the chick requirement which has been reported to be 0.9% of the ration (Almquist, '45). The poult requirement for total protein is approximately 1.2 (i.e., 24/20) times greater than the chick requirement. If the chick requirement for arginine were increased by this factor it would equal 1.08% arginine which is still only 80% of the amount required for poults. Thus the high requirement for arginine by the poult cannot be explained entirely by the higher requirement for total protein. The difference in the arginine requirement of chicks and poults is quite similar to that reported previously to exist for lysine. In the latter case, the poult requirement was found to be approximately 1.3% of the ration, compared with 0.9% for the chick (Grau, Kratzer and Asmundson, '46).

The high requirement of the poult for arginine indicates that it has very limited ability, if any, to synthesize arginine. In this respect the poult is similar to the chick and is in contrast to the rat which can grow at a suboptimal rate without a dietary source of arginine.

It is of interest to mention that in the third experiment, gains produced in groups on adequate levels of arginine were slightly greater than gains produced in a control group fed a practical poult starter.

#### SUMMARY

Poults were fed a purified type ration with casein, glycine, cystine, and various levels of arginine as the only source of amino acids. Approximately 1.35% arginine was required in the total ration to produce optimum growth.

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# FOLIC ACID, PARA-AMINOBENZOIC ACID AND ANTI-PERNICIOUS ANEMIA LIVER EXTRACT IN SWINE NUTRITION <sup>1</sup>

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Lindley and Cunha ('46) showed that the addition of inositol and biotin to the ration (purified) of the young pig did not increase growth or efficiency of feed utilization. However, they showed that biotin and inositol were needed by the pig when sulfathalidine was included in the ration at a 0.5% level. In their work with the pig it was postulated that inositol functioned indirectly by stimulating intestinal synthesis of biotin. A biotin deficiency syndrome similar to that observed by Lindley and Cunha ('46) as a result of feeding sulfathalidine in the ration was shown to occur when desiccated egg white, at a 30% level, was included in the ration of the pig (Cunha, Lindley and Ensminger, '46). Cartwright, Wintrobe and Humphreys ('46) produced an anemia in a pig, by including 2% of sulfasuxidine in the ration, which responded to a highly purified liver extract; however, they were not sure whether the anemia was due to a folic acid deficiency.

The experiment which is herein reported was undertaken to determine whether the addition of folic acid and para-

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aminobenzoic acid alone, or in combination with inositol and biotin, would be of any benefit, when added to a purified basal ration containing the 6 B-complex vitamins: thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline. In addition, a liver preparation (used in pernicious anemia in humans) was compared with folic acid for the pig. The effect of sulfathalidine administration on vitamin requirements of the pig was also studied.

#### EXPERIMENTAL

Eight-week old pigs were used in the lots where purified rations were fed. These pigs were farrowed by 4 Chester White gilts which had been bred to a Danish Landrace boar. The dams of these pigs were fed a ration of the following percentage composition during gestation and lactation: wheat 27, barley 27, cull peas 20, millrun 10, tankage 5, alfalfa meal 10, oyster shell 0.5, and iodized salt 0.5%.

The pigs were distributed equally into lots on the basis of weight, sex, and age. One pig from each of the 4 dams was placed in each of the 5 lots where purified rations were fed. The rations fed are shown in tables 1 and 2. The same pigs were used throughout the 2 phases of this experiment. During the first phase (7-week period) the effect on the nutrition of the pig of folic acid and para-aminobenzoic acid, alone and in combination with inositol, biotin, and a liver preparation<sup>2</sup> used in the treatment of pernicious anemia in humans, was investigated. During the second phase (5-week period) the effect of sulfathalidine on the need for these vitamins by the pig was determined.

The purified basal ration used in this experiment was the same as that reported previously by Heinemann, Ensminger, Cunha and McCulloch ('46). The ration consisted of casein<sup>3</sup> 26.1%, sucrose 57.7%, lard 11% and mineral mix<sup>4</sup> 5.2%.

<sup>2</sup> "Concentrated Solution Liver Extract, Parenteral" used for treatment of pernicious anemia. This was supplied by Lederle Laboratories, Pearl River, N. Y.

<sup>3</sup> Vitamin Test Casein GBI, manufactured by General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>4</sup> Same as used by Wintrobe ('39), and as modified by Heinemann et al. ('46).

Water soluble vitamins were fed (in mg per kg of live weight daily) as follows: thiamine 0.52, riboflavin 0.12, niacin 1.20, pantothenic acid 0.50, pyridoxine 0.20, and choline chloride 10.00; fat soluble vitamins were supplied (per pig daily) as follows: vitamin A, 5,000 I.U., vitamin E, 57 mg, vitamin K, 2 mg, vitamin C, 50 mg, and vitamin D, 700 I.U.

A modification of the "paired feeding technique" (Mitchell and Beadles, '30), was used in order to maintain the same feed intake by all pigs fed purified rations. Thus, the feed intake of the pigs was limited to the amount consumed by the pig with the least appetite — with the vitamins and the sulfonamide studied being the only variable among the lots of pigs. The pigs were fed in individual feeding stalls. To prevent coprophagy the pigs were kept on raised floors which were washed twice daily. At no time during the experiment was any tendency for the pigs to eat their feces observed.

To prevent rancidity and subsequent destruction of vitamins, the purified ration was mixed every third day and kept in an ice box. All vitamin solutions were stored under refrigeration. The required amount of the vitamins fed each pig was measured in calibrated pipettes and placed on top of the ration every other day (every fourth feeding) just before feeding time.

The pigs in lots VI and VII were fed natural grain rations. The pigs in lot VIA were of similar background and age as those fed purified rations. The rest of the pigs fed natural grain rations were 1 week younger and from Chester White sows fed the same ration as that fed the dams of the pigs on purified rations.

In lot VI, the natural (fish meal) ration consisted of wheat 20, barley 18.5, millrun 4.0, cull peas 16.0, soybean oil 6.0, fish meal (66.8% protein) 30.0, alfalfa meal (sun-cured) 5.0, and salt 0.5%. In lot VIA the pigs were self-fed this ration, whereas, in lot VIB the intake of feed was limited.

In lot VII, the natural (tankage) ration consisted of wheat 46.0, barley 35.0, tankage (dry rendered — 56.8% protein) 13.5, alfalfa meal (sun-cured) 5.0, and salt 0.5%. The amount

of feed fed these 6 pigs was the same and limited to the amount eaten by the pig with the least appetite. One-half of the pigs (those in lot VIIA) were given folic acid.

Each pig was bled weekly and blood was taken from the marginal ear vein for determination of hemoglobin, erythrocyte, leucocyte, and differential cell counts as well as reticulocyte studies.

Pipettes were filled from the flowing blood on the ear. Smears were prepared from the same source, the stagnant blood being wiped away as it gathered. Hemoglobin was determined according to the method of Evelyn ('36). Leucocytes were counted in 2% acetic acid diluent tinted with gentian violet. Erythrocytes were diluted with Hayem's solution. Smears for differential leucocyte counts were stained with Wright's stain and 200 cells were examined. Reticulocyte smears were stained with brilliant cresyl blue and counterstained with Wright's stain.

## RESULTS AND DISCUSSION

### *Growth studies*

The data in table 1 show that no appreciable beneficial effect on external appearance, growth, or efficiency of feed utilization was obtained when either folic acid or para-aminobenzoic acid was added alone or in combination with inositol and biotin to the purified basal ration. One pig (no. 330) which received the anti-pernicious anemia liver extract in addition to folic acid (lot 1B) made poor gains, became rough and scaly, and developed a brownish exudate throughout the surface of the body. Evidently, this pig (no. 330) reacted abnormally by developing an allergy-like condition in response to something present in the liver extract.<sup>5</sup>

Frequent scouring, during the first 5 weeks, was observed in the pigs on a restricted intake of the natural ration containing fish meal (lot VIB). The scouring accounts for the

<sup>5</sup> Dr. J. L. Rice, of Lederle Laboratories, saw this pig and stated that occasionally humans will react in a similar manner to the liver extract.

TABLE 1

*The effect of folic acid (FA), p-aminobenzoic acid (PAB), inositol (IN), biotin (B), and anti-pernicious anemia liver extract (LE) on growth.*

Type of ration	PURIFIED						NATURAL GRAIN			
	Basal <sup>1</sup> only	Basal <sup>1</sup> + PAB <sup>2</sup> + LE <sup>3</sup>	Basal <sup>1</sup> + FA <sup>4</sup>	Basal <sup>1</sup> + PAB <sup>2</sup> + FA <sup>4</sup>	Basal <sup>1</sup> + PAB <sup>2</sup> + IN <sup>5</sup> + B + FA <sup>4</sup>	Basal <sup>1</sup> + PAB <sup>2</sup> + IN <sup>5</sup> + B + FA <sup>4</sup>	30% fish meal in the ration		13.5% tankage in the ration	
							Ration self-fed	Amount of ration fed was limited	Folic acid added	Nothing added
Lot number	IA	IB	II	III	IV	V	VIA	VIB	VIIA	VIIIB
Number of pigs	4	2	4	4	4	4	3	3	3	3
Initial age, weeks	8	8	8	8	8	8	8	7	7	7
Number of weeks on trial	7	7	7	7	7	7	7	7	4	4
Av. initial weight, lbs.	34.7	30.5	32.7	33.5	32.5	32.0	42	31.3	36.6	39.6
Av. daily gain, lbs.	0.78	0.74	0.80	0.80	0.86	0.82	0.92	0.61	0.75	0.58
Av. daily feed consumption, lbs.	1.63	1.63	1.63	1.63	1.63	1.63	2.66	1.91	2.32	2.32
Lbs. of feed per lb. of gain	2.08	2.20	2.03	2.03	1.89	1.98	2.89	3.13	3.09	4.00

<sup>1</sup> Basal ration contained 6 B-complex vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline).

<sup>2</sup> Injected intramuscularly, in the gluteal muscle, every fourth day. It was injected at a rate of 0.067 ml daily (1 U.S.P. injectable unit) during the first 3 weeks. Following that period, the amount injected was doubled.

<sup>3</sup> Crystalline folic acid was fed in all lots at a level of 50 µg per 100 gm of feed.

<sup>4</sup> Para-aminobenzoic acid was fed in all lots at a level of 10 mg per 100 gm of feed.

<sup>5</sup> Inositol was fed in all lots at a level of 100 mg per 100 gm of feed.

<sup>6</sup> Biotin was fed in all lots at a level of 20 µg per 100 gm of feed.



poor gains and lowered efficiency of feed utilization obtained when comparing results on self-feeding versus limited feeding of the ration containing fish meal during the first 7 weeks of the experiment.

Supplementing with folic acid the natural ration containing tankage resulted in faster gains and in less feed being required per pound of gain. However, this effect was obtained only during the first 4 weeks of the experiment. In the next 5 weeks (table 2), folic acid did not improve the rate of gain. However, at the end of the 9-week feeding trial, the pigs fed folic acid were cleaner in appearance and had bigger appetites. It is possible that if the pigs fed folic acid had not been restricted in their feed intake, they would have eaten more and consequently outgained the pigs not being fed this dietary factor.

Occasional scouring, characterized by grayish feces, was obtained in all lots of pigs fed the purified rations during the first 7 weeks of the experiment. The scouring usually lasted from 36 to 48 hours and did not seem to have any effect on appetite. This scouring was observed even though, in lot V, all the known vitamins were being fed. No scouring was obtained with the pigs during the second 5 weeks of the experiment when sulfathalidine was included in the ration. However, the pigs fed the basal ration without sulfathalidine, continued to scour occasionally during the second 5 weeks of the experiment. Therefore sulfathalidine, fed at a level of approximately 0.65 gm per 10 pounds body weight, was effective in preventing a type of scours which occurred even when all known vitamins were included in the ration. Ferrin ('46) found that oral administration of sulfathalidine at a rate of 0.75 gm per 10 pounds of body weight was effective in stopping scours which developed in pigs fed methionine in addition to a natural ration. Scours in young pigs fed natural rations have been reported by Ross et al. ('44), Fairbanks et al. ('44) and Whitehair et al. ('46). Whether there is any relation between the scours observed by these workers and

TABLE 2

The effect of sulfathalidine (ST) on the response of the pig to folic acid (FA), p-aminobenzoic acid (PAB), inositol (IN), biotin (B), and vitamin E (VE).

Type of ration	NATURAL GRAIN									
	PURIFIED					30% fish meal in the ration				
	Basal	Basal + ST <sup>1</sup>	Basal + VE + FA	Basal + PAB + FA	Basal + PAB + IN + B	Basal + PAB + IN + B + FA	Ration self-fed	Amt. of ration fed was limited	Folic acid added	Nothing added
Ration fed	Basal	Basal + ST <sup>1</sup>	Basal + VE + FA <sup>2</sup> + ST	Basal + PAB + FA + ST	Basal + PAB + IN + B + ST	Basal + PAB + IN + B + ST + FA	Same as above	Same as above	Same as above	Same as above
Lot number	IA	IAA	IB	II	III	IVA	IVB	V	VIA	VIB
Number of pigs	2	2	2	4	4	2	2	4	3	3
Initial age, weeks	15	15	15	15	15	15	15	15	15	11
Number of weeks on trial	5	5	5	5	5	5	5	5	5	5
Av. initial weight, lbs.	74.0	72.5	75.5	72.0	72.5	75.0	74.0	72.2	86.3	61.0
Av. daily gain, lbs.	1.47	1.44	1.19	1.46	1.39	1.42	1.44	1.43	1.43	1.07
Av. daily feed consumption, lbs.	2.83	2.83	2.83	2.83	2.83	2.83	2.83	2.83	6.07	3.33
Lbs. of feed per lb. of gain	1.93	1.96	2.37	1.94	2.03	1.99	1.96	1.97	4.24	3.11

<sup>1</sup> Sulfathalidine was fed at a level replacing 0.5% of the ration.

<sup>2</sup> Folic acid was fed in all lots at a level of 100 µg per 100 gm of ration during last 4 weeks. During first week it was fed at level of 50 µg per 100 gm of ration.

<sup>3</sup> Biotin, inositol, and para-aminobenzoic acid were fed at same levels indicated in table 1.

that obtained in this work with purified rations is difficult to say.

A small amount of blood was frequently noted in the feces of the pigs fed purified rations. Wood in the feces, as a result of fence chewing, was found with all the pigs fed purified rations. No fence chewing or blood in the feces was observed with the pigs fed the natural grain rations. In an effort to test whether a lack of sufficient phosphorus might be responsible for the fence chewing, an extra 10 gm of  $\text{CaHPO}_4$  were given each pig daily for 10 days. This was followed by increasing the total mineral intake of the ration from 5.2 to 7.2% for 21 days. The addition of the extra minerals seemed to alleviate, to a small extent, the fence chewing but did not eliminate it. Fence chewing has consistently been observed at this station with young pigs fed purified rations.

Since the addition of either folic acid or para-aminobenzoic acid to the basal ration for a 7-week period was of no benefit on gains or efficiency of feed utilization, it was assumed that possibly the pig may be able, through the action of intestinal bacteria, to synthesize enough of these 2 vitamins for those needs. However, there is the possibility that enough of these 2 vitamins may have been stored during the suckling period of these pigs to last them through this experiment. In order to test whether these 2 vitamins were being synthesized, sulfathalidine which has been used to inhibit the growth of intestinal bacteria and hence vitamin synthesis with the pig (Lindley and Cunha, '46) was used.

The data in table 2 show that the addition of sulfathalidine to the purified basal ration for a 5-week period had no effect on the need of the pig for either folic acid or para-aminobenzoic acid as measured by the rate of gain and efficiency of feed utilization. Among all the lots of pigs receiving purified rations, there was little or no difference in gains except in lot 1B where the anti-pernicious anemia liver preparation was fed in addition to folic acid. In this lot, 1 pig (no. 330) accounted for the poor gains. The other pig in this lot gained as well as the pigs in the other lots fed purified rations. The

exudate which pig no. 330 exhibited at the end of the experiment was not as severe as it was at the end of the first 7 weeks. Pig no. 330 developed cracked feet by the end of the experiment which were typical of those observed on a biotin deficiency with the pig by Lindley and Cunha ('46) and Cunha et al. ('46). After the third week, pig no. 330 began losing some hair on the posterior part of the ham and displaying a sensitivity in the feet and a tendency to lie down and remain inactive. All 4 feet became cracked and bled occasionally. The pig also displayed considerable hypersensitivity when forced to move. The hair loss and cracked feet are typical of a biotin deficiency. The other pig in this lot also had 4 cracked feet and a slight hair loss on the posterior part of the ham. However, the most marked biotin deficiency symptoms were obtained with pig no. 330.

In lot IAA (basal + sulfathalidine) 1 pig displayed cracks in the feet typical of a biotin deficiency. The other pig in this lot had a slight hair loss on the posterior part of the ham. In lot II (basal + folic acid) all 4 pigs displayed slight to moderately cracked feet typical of a biotin deficiency. Two pigs displayed a slight hair loss on the posterior part of the ham. In lot III (basal + PAB) 2 pigs displayed moderately cracked feet and 1 of these pigs had a slight hair loss on the posterior part of the ham. All pigs in lots IA, IVA, IVB, and V were normal in appearance with regard to hair coat and feet. This substantiates the work of Lindley and Cunha ('46), which showed that biotin in the ration would prevent the symptoms discussed above. The biotin deficiency symptoms exhibited in this work were very mild as compared to those observed previously by Lindley and Cunha ('46). This may be due to 2 things: (1) In the previous work lighter pigs were used and sulfathalidine was fed after the pigs had previously been fed sulfaguanidine for 5 weeks, and (2) the pigs were fed sulfathalidine for 6 weeks in the previous work as compared to 5 weeks in this trial. Miller ('45) showed that sulfathalidine fed to rats over a long period of time caused nutritional deficiency symptoms which were corrected by the feeding of

biotin and folic acid. Cartwright, Wintrobe and Humphreys ('46) were able to produce an anemia in the pig which responded to a highly purified liver extract. However, the anemia was produced after feeding 2% of sulfasuxidine in the ration for an extended period of time; they were not sure that the anemia was due to a folic acid deficiency.

The data in table 2 show that the pigs fed a limited amount of the natural ration containing fish meal were very efficient in utilizing their feed. During the second 5-week period, the pigs fed a limited amount of the fish meal ration required 3.11 pounds of feed per pound of gain whereas the pigs self-fed the same ration required 4.24 pounds of feed per pound of gain.

### *Studies of the blood*

*Hemoglobin.* The data in table 3 show that during the first 7-week period the addition of folic acid to the basal purified ration was of some help in hemoglobin formation. The addition of para-aminobenzoic acid to the basal ration resulted in slightly higher hemoglobin values. Those obtained with the pigs fed the natural grain rations (lots VI and VII) are somewhat in line with others reported in the literature by Kernkamp ('32), Croft and Moe ('32) and Nordskog et al. ('44).

It is of interest to note that the addition of folic acid to the basal purified ration resulted in higher hemoglobin values than those obtained with the pigs fed the natural grain rations. Since the natural ration containing 30% fish meal may be considered very well balanced nutritionally, one might wonder whether many supposedly well-balanced natural grain rations really support maximum hemoglobin formation. The question might also be raised as to whether the pigs fed the basal ration plus folic acid were better off nutritionally since their hemoglobin values were a little higher than those of the pigs self-fed the natural ration containing 30% fish meal.

The addition of folic acid alone to the basal purified ration (lot II) stimulated hemoglobin formation. However, when folic acid was added along with the liver extract (lot IB) or

other vitamins (lot V), it did not stimulate hemoglobin formation. This finding is difficult to explain. It is possible that it may have been caused by some vitamin imbalance. Suggestions of a possible imbalance of vitamins were obtained by Cunha et al. ('43) with rats, and by Cunha et al.

TABLE 3

*The effect of folic acid (FA), p-aminobenzoic acid (PAB), inositol (IN), biotin (B), and anti-pernicious anemia liver extract (LE) on hemoglobin level of pigs.*

LOT NO.	RATION FED		BLOOD HEMOGLOBIN <sup>1</sup> (GM/100 ML)		
			First 7 weeks		Next 5 weeks
			No sulfonamide added	No sulfonamide added	Sulfathalidine added
IA	Basal		12.51	12.89	13.60
IB	Basal + FA + LE		12.33		13.23
II	Basal + FA		14.18		13.90
III	Basal + PAB		13.49		13.01
IVA	Basal + PAB + IN + B		12.77		13.40
IVB	Basal + PAB + IN + B + LE				13.24
V	Basal + PAB + IN + B + FA		12.17		13.09
VIA	Natural ration	Self-fed	12.57	13.05	
VIB	with fish meal	Limited amount fed	12.52	13.13	
VIIA	Natural ration	Folic acid added	11.47	11.32	
VIIB	with tankage	Nothing added	11.55	11.54	

<sup>1</sup> The hemoglobin values are an average figure of all the times the pigs were bled.

('44) with sows. With both rats and sows, there were many instances where the addition of 1 vitamin or a combination of vitamins gave poorer results than when no vitamins were added to the basal ration being studied.

Of interest is the fact that the hemoglobin values were about the same with the pig self-fed as compared with those fed a limited amount of the same natural grain ration containing fish meal. The addition of folic acid to the natural grain ration containing tankage had no effect in stimulating hemoglobin formation although it stimulated growth to a slight extent during the first 4 weeks of the experiment. The addition of sulfathalidine to the purified rations did not have any effect in lowering hemoglobin values. This may have been caused by too low a level being fed, or the feeding of sulfathalidine not being continued for a long enough period.

*Erythrocytes.* Weekly averages of all groups of pigs showed erythrocyte counts in the range of 6 to 10 millions per mm<sup>3</sup>. Kernkamp ('32) reported an average of about 7 million erythrocytes in pigs 40 to 80 days of age. Figures reported by Kolmer and Boerner ('41) show a range of 5 to 9 million erythrocytes per mm<sup>3</sup> for swine, although the ages of their pigs were not given. It would thus appear that neither anemia nor polycythemia was produced in pigs in this experiment since their erythrocyte counts were within normal ranges. No appreciable differences in number of erythrocytes were obtained between the various groups of pigs. Excessive numbers of immature or abnormal erythrocytes were not observed.

*Reticulocytes.* The reticulocyte counts in the various groups of pigs averaged from 0.3–2.6% of the erythrocytes. The reticulocyte numbers were consistently lower during the period sulfathalidine was fed than they were in the same group during the first part of the experiment. Coffin ('46) reports a reticulocyte range of 0.5–4.5% in swine. It is assumed that the reticulocyte counts in this experiment were within a normal range.

*Leucocytes.* Weekly averages of all groups of pigs showed leucocyte counts in the range of 10,000 to 30,000 per mm<sup>3</sup>. Kernkamp ('32) reported an average leucocyte count of about 21,600 in pigs 54 to 80 days old. From Kernkamp's work it would appear that young pigs have a relatively high leucocyte count, as was found in this work. While some of the leucocyte counts were high, there was no appreciable difference among the various groups, and the counts were regarded as being within normal range.

During the last 3 or 4 weeks of the experiment there developed a moderate relative lymphocytosis and neutropenia, which was less marked with the natural grain ration fed the pigs in lots VI and VII. It may be that this difference was due to some factor or factors contained in the natural rations and not contained in the purified rations. There was no appreciable difference among the groups of pigs fed purified rations in this regard.

#### SUMMARY AND CONCLUSIONS

During a 7-week trial, no beneficial effect on external appearance, growth, or efficiency of feed utilization was obtained when either folic acid or para-aminobenzoic acid was added alone or in combination with inositol and biotin to a purified basal ration which contained the 6 B-complex vitamins—thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and choline.

The addition of folic acid or para-aminobenzoic acid alone to the basal purified ration stimulated hemoglobin formation to a small extent. However, when folic acid was added to the purified basal ration in addition to the liver extract or other vitamins, it did not stimulate hemoglobin formation. It is possible that this may have been due to some vitamin imbalance. The addition of folic acid alone to the basal purified ration resulted in higher hemoglobin values than those obtained with pigs fed a natural grain ration containing 30% fish meal. The provision of folic acid to pigs fed a natural grain ration containing tankage did not have any effect on hemoglobin



formation although it caused some stimulation of growth during the first 4 weeks and caused the pigs to be cleaner in appearance and have bigger appetites at the end of the experimental period. This may also mean that even though folic acid does not help growth with a purified ration, it may, under certain conditions, promote growth with natural grain rations.

One pig, being injected with the anti-pernicious anemia liver extract, reacted to it abnormally. The pig made poor gains, developed rough and scaly skin, and a brownish exudate throughout the surface of the body.

Sulfathalidine, fed at a level of approximately 0.65 gm per 10 pounds of body weight, was effective in preventing the scours, characterized by a grayish feces, which occurred periodically with the pigs fed purified rations even in the group where all the known vitamins were fed.

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# THE SPARING ACTION OF PROTEIN ON THE PANTOTHENIC ACID REQUIREMENT OF THE RAT

## II. URINARY AND FECAL EXCRETION OF PANTOTHENIC ACID <sup>1</sup>

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### TWO FIGURES

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The sparing action of high protein (casein) diets on the pantothenic acid requirement of the rat (Nelson and Evans, '45) was shown by the increased growth and survival of rats deficient in pantothenic acid from birth or from weaning. This sparing action may be due to (1) an "absolute" decrease in the requirement for the vitamin; (2) a relative decrease because of decreased vitamin loss by urinary excretion; or (3) an increased intestinal synthesis of the vitamin. To investigate the latter 2 possibilities the urinary and fecal excretion of pantothenic acid has been determined for deficient rats maintained on purified diets varying from 24% to 64% casein.

The present study shows that the fecal excretion of the vitamin was variable and a relation to the dietary protein level not apparent. This would seem to exclude the third view that increased intestinal synthesis of the vitamin occurs. On the

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<sup>2</sup> General Mills Fellow.

other hand, the urinary vitamin excretion was definitely observed to increase with the protein intake. The second explanation that a decreased vitamin loss by urinary excretion occurs is, therefore, erroneous. The data here presented, therefore, indicate instead that there is an unexplained "absolute" decrease in the rat's requirement for pantothenic acid as such when higher protein intake occurs.

The marked increases observed in urinary vitamin excretion prompted a reexamination of the pantothenic acid content of various purified and "vitamin-free" caseins, all of which were found to contain significant amounts of pantothenic acid. When the calculated amount of pantothenic acid present in the high-protein diets was added to the high-carbohydrate diet, such supplemented diets were still not equal in sparing action to the high-protein diets on which littermates were maintained.

#### EXPERIMENTAL PROCEDURES

Twenty-one day old rats, littermates of the Long-Evans strain, were divided into equivalent groups, balanced according to body weight, and placed in individual cages with screens. Males were used for all experiments except the first one. The animals were examined and weighed every 5 days; those in poor condition were checked daily.

The purified pantothenic acid-deficient diets used varied only in their proportions of carbohydrate and protein. The basal diet (high-carbohydrate) contained 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil,<sup>3</sup> and 4% salts.<sup>4</sup> The high-protein diet contained 64% casein and 24% sucrose with the remainder of the constituents identical. Both diets contained the following crystalline B vitamins per kilogram of diet: 5 mg thiamine-HCl, 5 mg pyridoxine-HCl, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid, 400 mg inositol, and 1 gm choline chloride. Each rat received weekly a fat-soluble vitamin mixture con-

<sup>3</sup> Crisco.

<sup>4</sup> Salts no. 4 of Hegsted et al. ('41).

taining 325 mg corn oil,<sup>5</sup> 400 U.S.P. units vitamin A, 58 Chick Units vitamin D, and 3 mg alpha-tocopherol.

The microbiological assay method of Strong et al. ('41) as modified by Neal and Strong ('43) and by Strong ('44) was used for pantothenic acid analyses. Urine and feces were collected for a 24-hour period and pooled for each dietary group. Individual metabolism cages were used for each rat and every precaution taken to eliminate contamination of urine with the feces. This was especially important because the pantothenic acid content of feces was considerably higher than that of the urine. Food was not given to the rats during the 24-hour collection period because of the difficulty in preventing scattering of the diet with the subsequent contamination of urine and feces. Also, it was felt that food intake during this period would introduce an additional variable into both the urinary and fecal excretion.

## RESULTS

### *Growth and survival*

Table 1 summarizes the data on growth and survival of deficient rats maintained on the 24% and on the 64% casein diets. Growth and survival on the high protein level were in striking contrast to those on the lower level. Furthermore, the superiority of the high-protein diet increased with age. Both growth and survival on the 64% casein diet were slightly but significantly better by 60 days of age and markedly better by 90 days of age. Few animals maintained on the 24% casein diet survived 120 days (only 21% under the experimental conditions), whereas animals maintained on the 64% casein diet survived indefinitely once they were past the critical age of 50-70 days. Animals have been maintained as long as 12 months on the high protein diet deficient in pantothenic acid, their body weight and general condition improving with each month. It will be noted that the few deaths on the 64% level occurred, with only a single exception, before 60 days.

<sup>5</sup> Mazola.

Apparently 1 or 2 rats in each series cannot become adjusted to the high protein diet in time for the sparing action to be effective.

The typical symptoms of pantothenic acid deficiency observed in the groups receiving the 24% casein level (greying, porphyrin deposition, dermatitis, and hemorrhagic adrenals) were ameliorated at the 64% level. Greying occurred on the

TABLE 1

*Effects of dietary protein level on growth and survival of pantothenic acid deficient rats.*

% CASEIN	NO OF RATS	AV WT. DAY 21	AV WT. DAY 60	SURVIVAL DAY 60	AV WT DAY 90	SURVIVAL DAY 90	AV. WT. DAY 120	SURVIVAL DAY 120
		gm	gm	%	gm	%	gm	%
24	13 ♀	47	84	54	93	8	...	0
	10 ♂	48	110	80	110	50	94	10
	12 ♂	47	100	58	120	42	132	25
	15 ♂	48	94	67	118	40	137	40
	10 ♂	48	102	70	122	10	146	10
	17 ♂	50	111	73	128	53	136	29
Averages	77 ♀							
	and ♂	48	101	68	120	35	134	21
64 <sup>1</sup>	12 ♀	48	115	83	170	83	202	83
	10 ♂	47	141	90	234	90	300	90
	12 ♂	47	124	92	205	83	255	83
	5 ♂	48	112	100	170	100	200	100
Averages	39 ♀							
	and ♂	47	124	90	197	87	243	87

<sup>1</sup> The animals maintained on the 64% casein diet are littermates of the corresponding first 4 groups on the 24% casein diet. The additional animals on the 24% level are included to show the variation in growth and survival on this diet.

high-protein level at the same time it was observed on the lower level (45-65 days of age) but in the majority of animals maintained on the high protein level the original fur color began to return between 90 and 120 days of age. Spontaneous cures of greying have been reported for rats receiving sub-optimal levels of pantothenic acid by Henderson et al. ('42) and by Unna and Richards ('42). Porphyrin deposition and

dermatitis were not observed at the high protein level; however, hemorrhagic adrenals were found in the few rats that died at an early age.

### *Urinary and fecal excretion of pantothenic acid*

It is evident from the data summarized in figure 1 that the urinary excretion of pantothenic acid was significantly higher for rats on the 64% casein diet than for those on the 24% casein diet. Urinary excretion on the high casein level increased directly with age and paralleled the increased growth and survival observed on this diet. This increasing superiority of the high protein diet with age may be due to the fact that the pantothenic acid requirement of the rat decreases with age (Unna and Richards '42). The daily urinary excretion of pantothenic acid for 56 rats maintained on the 64% casein diet averaged 6.0  $\mu\text{g}$ , beginning with 3.3  $\mu\text{g}$  (day 30-60) and 4.6  $\mu\text{g}$  (day 60-90) up to 6.7  $\mu\text{g}$  (day 90-120) and 7.1  $\mu\text{g}$  (day 120-165). Additional determinations carried out on 21 older rats, 225-285 days of age (not shown on the figure), averaged 10.7  $\mu\text{g}$  daily. In contrast, the daily urinary excretion of pantothenic acid for 45 rats maintained on the 24% casein diet averaged 0.9  $\mu\text{g}$ , with corresponding figures for the same age groups of 0.8  $\mu\text{g}$ , 0.8  $\mu\text{g}$ , 1.1  $\mu\text{g}$ , and 1.1  $\mu\text{g}$ , respectively. The vitamin excretion on this diet was similar but slightly higher (as would be expected) than the values reported by Henderson et al. ('42) for pantothenic acid deficient rats maintained on an 18% casein diet.

The fecal excretion of pantothenic acid varied markedly, regardless of the casein level. Up to 90 days of age, at which time growth and survival on the high protein level are already markedly better than on the lower level, there was no significant difference in fecal excretion for the 2 protein levels.<sup>a</sup>

<sup>a</sup> Recently, Wright and Skeggs ('46) have reported that the fecal excretion of the B vitamins (including pantothenic acid) varied directly with the protein level when diets supplemented with 8 B vitamins but furnishing deficient or suboptimal amounts of casein (5%-18%) were used. It may be noted that the lowest casein level used in the present study was 24%.



After 90 days of age the high protein group tended to excrete more pantothenic acid in their feces. This tendency is shown in the averages for the dietary groups and for the different

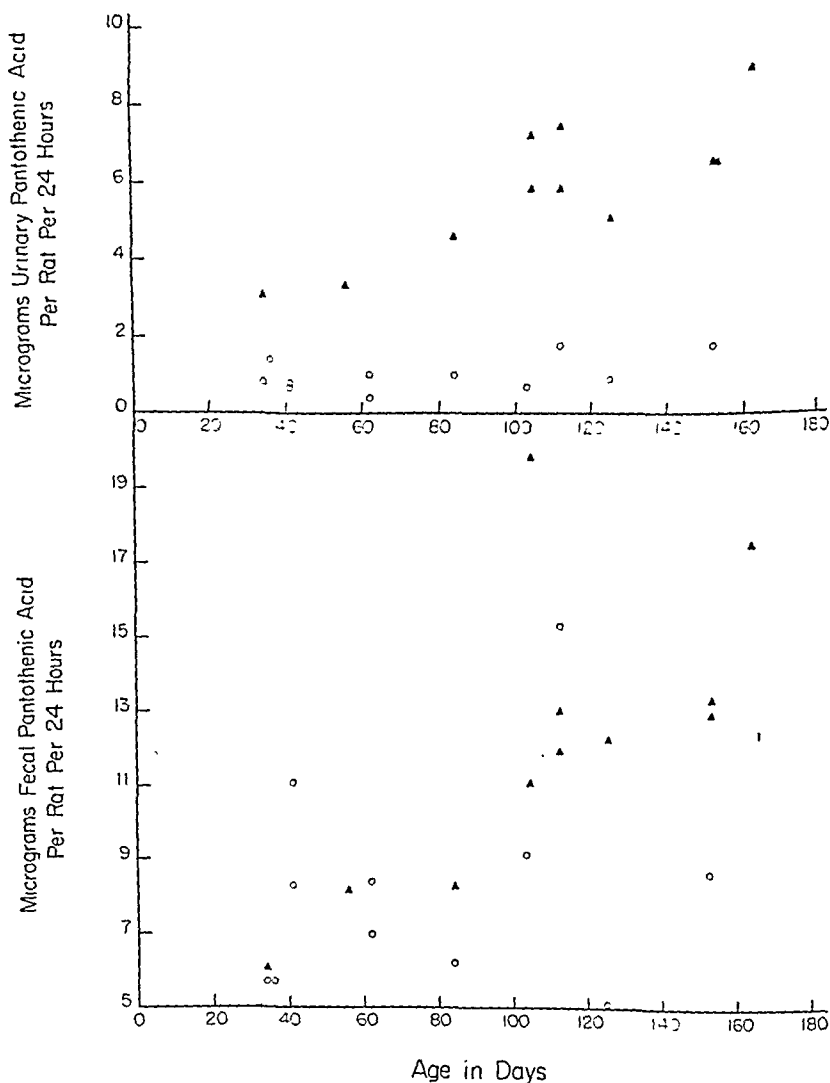


Fig. 1 Urinary and fecal pantothenic acid excretion by pantothenic acid deficient rats maintained on diets containing 24% and 64% casein.  $\circ$  = 24% casein diet,  $\blacktriangle$  = 64% casein diet.

age groups on the diet. Fifty-eight rats maintained on the 64% casein diet averaged 12.4  $\mu\text{g}$  fecal pantothenic acid per 24 hours, beginning with 7.2  $\mu\text{g}$  (day 30-60) and 8.3  $\mu\text{g}$  (day 60-90) to 14.1  $\mu\text{g}$  (day 90-120) and 14.2  $\mu\text{g}$  (day 125-165). In addition, determinations carried out with 23 older animals, 225-285 days of age (not shown on the figure) averaged 20.9  $\mu\text{g}$  daily. On the 24% casein diet 45 rats averaged 8.0  $\mu\text{g}$  fecal pantothenic acid per 24 hours and the corresponding figures for the same age groups were 7.8  $\mu\text{g}$ , 7.5  $\mu\text{g}$ , 11.5  $\mu\text{g}$ , and 6.5  $\mu\text{g}$ , respectively. Few determinations were carried out on rats maintained on the 24% casein level over 120 days of age because of the small number of animals surviving this period.

Mention has been made of a possible effect of food intake on fecal excretion. Fecal excretion of pantothenic acid was measured on a group of 9 rats maintained on the 64% casein diet and 160-165 days of age. When fasted the fecal pantothenic acid per 24 hours averaged 17.5  $\mu\text{g}$  (range 9-24). When given food the fecal pantothenic acid over a period of 5 days averaged 28.2  $\mu\text{g}$  (range 15-48). The averages for successive 24- or 48-hour collection periods were 28.2  $\mu\text{g}$  (range 17-34), 32.9  $\mu\text{g}$  (range 25-39), and 25.9  $\mu\text{g}$  (range 15-48). The variation in fecal excretion for individual rats during these periods were as follows: 15-26, 17-28, 18-25, 18-33, 19-36, 24-48, 25-34, 25-38, and 27-39 or a difference of 7-24  $\mu\text{g}$  between the minimum and maximum values for any individual rat. These figures are given to indicate the extreme variability in fecal excretion, especially when the animals are given food. This marked variability is one reason for pooling fecal samples for dietary groups.

The data given on fecal excretion of pantothenic acid at the 2 protein levels do not indicate that increased intestinal synthesis of the vitamin occurred at the high protein level during the first 90 days of age. Although the fecal excretion at the high protein level increased with age, this may be due to the increase in body weight and correspondingly in size of cecum (Taylor et al., '42) rather than to the protein level

per se. The data given on urinary excretion clearly show an increase in vitamin excretion at the high protein level. There is, obviously, no relative decrease in the pantothenic acid requirement because of a decreased vitamin loss by urinary excretion on this diet.

### *Effect of intermediate protein levels*

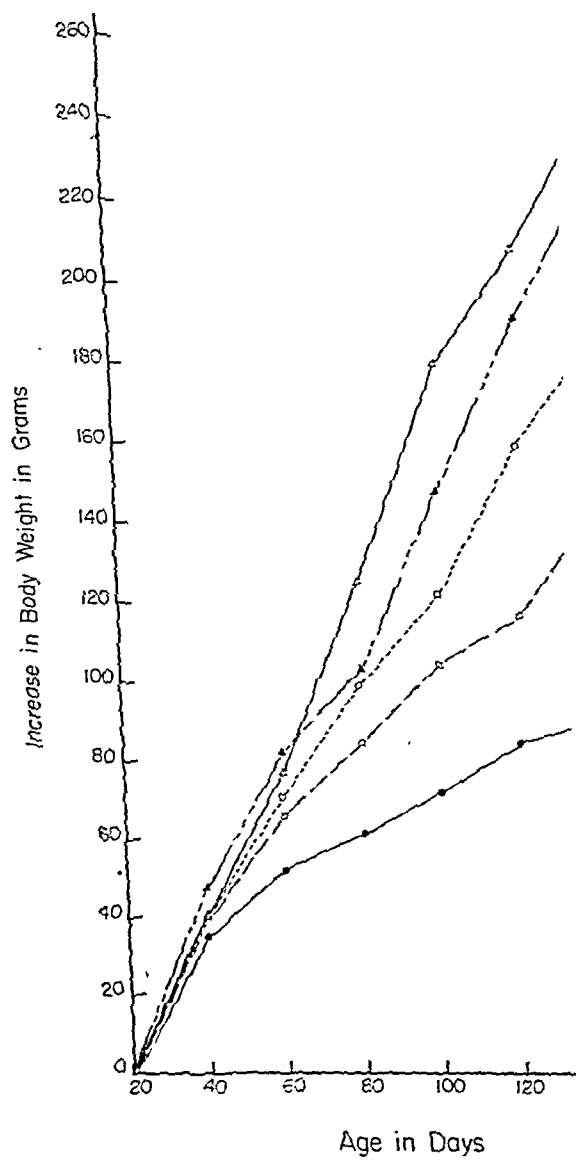
The marked differences in growth, survival and urinary excretion at the 2 casein levels, 24% and 64%, suggested the desirability of testing the intermediate levels of casein. Twelve litters of 21-day-old male rats were divided into equivalent groups and placed on the deficient diets containing 24%, 34%, 44%, 54%, and 64% casein, with corresponding decreases in the percentage of sucrose. The rats were observed until 150 days of age. Urinary and fecal pantothenic acid were measured during the latter half of the experimental period, i.e., the ninth, twelfth, thirteenth and nineteenth weeks of the experimental period.

Growth (fig. 2), survival and urinary pantothenic acid (table 2) were proportional to the protein level. In agreement with the data on fecal excretion for the 24% and 64% casein diets, there was no correlation between fecal pantothenic acid and the protein level. The sparing action of high casein diets, as judged by growth, survival and urinary excretion of pantothenic acid, was directly proportional to the casein level of the deficient diets.

### *Analysis of casein*

The marked increase in urinary excretion of pantothenic acid by rats maintained on the higher protein levels prompted a reexamination of the pantothenic acid content of the casein used in the purified diets and of other types of purified caseins. Microbiological assays using water extraction (and heat sterilization) had previously indicated that 3 types of casein prepared in the laboratory, namely acid-washed, ether-extracted, and alcohol-extracted caseins, contained insignificant

# PANTOTHENIC ACID REQUIREMENT OF



amounts of pantothenic acid (0.1 to 0.3  $\mu\text{g}$  per gm casein). Enzymatic digestion with clarase revealed the presence of considerably greater amounts of "bound" pantothenic acid in all samples of purified casein tested. The amount varied with the type of purification and with different batches in which the same purification procedure was used, i.e., alcohol-extracted casein, 2.8, 1.8, and 1.5  $\mu\text{g}$  per gm; acid-washed casein 2.8 and 1.9  $\mu\text{g}$ ; ether-extracted casein 2.0 and 1.8  $\mu\text{g}$ ; GBI casein 1.3  $\mu\text{g}$ ; and SMACO casein 0.9 and 0.7  $\mu\text{g}$ . Each value given is the average of 2 to 3 determinations on the

TABLE 2

*Effect of dietary protein level on pantothenic acid excretion and survival of deficient rats.*

% CASEIN	URINARY PANTOTHENIC ACID $\mu\text{g}/\text{RAT}/24 \text{ HRS.}^1$		FECAL PANTOTHENIC ACID $\mu\text{g}/\text{RAT}/24 \text{ HRS.}^1$		% SURVIVAL			
	Average	Range	Average	Range	Day 60	Day 90	Day 120	Day 150
24	1.6 (6) <sup>2</sup>	1.1-1.8	11.4 (7)	4.0-15.4	58	42	25	17
34	1.8 (16)	1.6-2.0	12.4 (15)	8.1-19.3	75	50	50	50
44	3.5 (13)	2.7-3.7	9.1 (14)	6.7-15.5	87	67	50	50
54	5.8 (13)	2.4-6.2	12.9 (17)	4.0-15.5	100	75	67	67
64	6.6 (25)	3.0-7.5	12.2 (26)	9.0-13.2	92	83	83	83

<sup>1</sup> These values are the averages of determinations carried out on the ninth, twelfth, thirteenth and nineteenth weeks of the experiment. Twenty-four-hour samples were pooled for each dietary group. Numbers in parentheses refer to the number of rats on which the average is based.

<sup>2</sup> This value is higher than usual because the determinations were carried out on the few rats surviving during the later half of the experiment.

same sample. These values are approximately twice as high as those reported by Cannon et al. ('45) on similar purified caseins (the enzyme used by Cannon is not specified) but the variability between different batches of casein and slight variations in the purification procedure would be sufficient to explain the differences. The presence of pantothenic acid in a "bound" or combined state in many biological materials and methods for releasing the vitamin from the combined state have been reported by several investigators (Pennington, Snell and Williams, '40; Waisman et al., '42; Wright, '43). It may be mentioned that enzymatic digestion of urine and feces did not increase the pantothenic acid values so that

presumably all pantothenic acid excreted by the rat is in a "free" or uncombined state.

*Effect of 24% casein diets supplemented with low levels of pantothenic acid*

Since a purified casein<sup>7</sup> free of pantothenic acid was not available, low levels of pantothenic acid equivalent to or greater than the amount contained in the casein of the high protein diets, were added to the 24% casein diet. All diets were prepared from 1 batch of casein and 4 different analyses (with and without ether extraction) were carried out on each diet. Three casein levels (24%, 44% and 64%) were used and in addition 2 supplemented diets containing 24% casein. One supplemented diet contained slightly more pantothenic acid than either of the 2 higher protein diets; the other contained twice as much pantothenic acid as the 44% casein diet and slightly less than twice as much as the 64% casein diet. The diets together with their pantothenic acid content are as follows: 24% casein diet containing 0.3  $\mu\text{g}$  pantothenic acid per gm diet; 2 supplemented diets, 24% casein, with 0.9 and 1.4  $\mu\text{g}$ , respectively; 44% casein diet, 0.7  $\mu\text{g}$ ; and 64% casein diet, 0.8  $\mu\text{g}$ .

Five litters of 21-day-old male rats were used and observed until 120 days of age. Urinary and fecal excretion of pantothenic acid was measured at intervals throughout the entire experimental period, i.e., second, fifth, ninth, twelfth and fifteenth weeks.

The data in table 3 show, as expected, that growth<sup>8</sup> and urinary excretion of pantothenic acid by rats maintained on

<sup>7</sup> Recent studies have indicated the possibility of freeing beef blood fibrin from traces of pantothenic acid by washing with water or dilute acid. Further experiments are in progress.

<sup>8</sup> The groups in this experiment were too small (5 rats per group) to obtain much significant data on survival. It may be noted that all other experimental groups reported have contained 10-15 rats per group. 100% of the animals survived 90 days on the 64% casein diet and on the corresponding 24% casein supplemented diet (0.9  $\mu\text{g}$  per gm diet); 60% of the animals survived the same period in the remainder of the groups.

the 24% casein diets with or without supplements were proportional to the pantothenic acid content of the diet. This is in agreement with the studies of Henderson et al. ('42). However, growth and urinary pantothenic acid of littermates maintained on the higher protein levels (44%<sup>9</sup> and 64%)

TABLE 3

*Effect of dietary protein level and low levels of pantothenic acid on vitamin excretion and growth of deficient rats.*

EXPTL GROUP		URINARY PANTOTHENIC ACID $\mu\text{G}/\text{RAT}/24 \text{ HRS}^1$		FFCAL PANTOTHENIC ACID $\mu\text{G}/\text{RAT}/24 \text{ HRS}^1$		AV. BODY WT.—GM		
Casein %	Panto thenic acid content $\mu\text{G}/\text{gm}$ diet					Day 60	Day 90	Day 120
		Average	Range	Average	Range			
24	0.3	0.7 (14)	0.6–0.9	4.4 (14)	3.2–5.7	79	94	98
24	0.9	1.6 (19)	1.1–2.1	6.8 (19)	5.7–8.4	91	135	163
24	1.4	1.9 (18)	0.8–3.9	5.6 (18)	3.8–10.9	95	144	191
44	0.7	2.2 (13)	1.6–2.8	7.3 (13)	3.9–10.4	107	158	185
64	0.8	4.5 (25)	3.1–5.9	10.9 (25)	6.1–19.9	112	170	200

<sup>1</sup> Values are averages for determinations carried out on the second, fifth, ninth, twelfth and fifteenth weeks of the experiment. Twenty-four-hour samples were pooled for each dietary group. Numbers in parentheses refer to the number of rats on which the average is based.

were markedly greater than on the 24% casein diet with the corresponding pantothenic acid content (0.9  $\mu\text{g}$  per gm diet). When the pantothenic acid content of the 24% casein diet was double or practically double that of the higher protein levels, growth and urinary pantothenic acid were approximately equal to that produced by the 44% casein diet but were still not equivalent to the results obtained with the 64% casein diet. Therefore, it was concluded that the pantothenic acid content of the casein used in the purified deficient diets does not account for all of the sparing action of the

<sup>9</sup> It may be mentioned that every weekly determination of urinary pantothenic acid for rats maintained on the 44% casein diet was higher than the value obtained from the corresponding 24% casein supplemented diet (0.9  $\mu\text{g}$  per gm diet).

high casein diets, although it is responsible for part of the protective action observed.

In regard to fecal excretion of pantothenic acid, the averages showed an apparent increase proportional to the casein level. This was not considered significant in view of the marked variability in fecal excretion (see fig. 1). The tendency of the fecal excretion to increase with age on the high protein levels may or may not result in an increased average value (compare tables 2 and 3).

#### DISCUSSION

The data reported in this paper indicate that neither increased intestinal synthesis as measured by fecal excretion nor a decreased vitamin loss by urinary excretion are responsible for the sparing action of high casein diets on the pantothenic acid requirement of the rat. Furthermore, the pantothenic acid content of the casein used in the purified diet, while exercising an effect, cannot account for the protective action of such diets. Therefore, it would appear that there is a real decrease in the pantothenic acid requirement of the rat when higher protein intake occurs.

It is, of course, possible that some component of casein may replace or be converted to pantothenic acid in the body in a manner similar to the tryptophane-niacin interrelationship (Krehl et al., '46). This would explain the increased urinary excretion of pantothenic acid on the high casein diets. It is also possible that this increased urinary pantothenic acid excretion may be merely a reflection of the increased food intake on such diets, an increase caused by some factor(s) concerned in the efficient utilization of pantothenic acid. Such a factor might be a known or unknown component of casein as previously suggested (Nelson and Evans, '45), e.g., amino acids, phosphorus, or unknown factors similar in their action to biotin and folic acid which are reported (Wright and Welch, '43, '44) to be necessary for pantothenic acid utilization. It has also been suggested recently (Wright and Welch, '46)



that the necessity of a protein substrate for pantothenic acid combination in blood and tissues may explain the sparing action of high protein diets. On the other hand, the observed sparing action may actually be due to the decreased level of carbohydrate metabolism. Further investigations of these possibilities are in progress.

#### SUMMARY

Studies of urinary and fecal excretion of pantothenic acid by deficient rats maintained on purified diets differing in casein level gave the following results:

1. Urinary pantothenic acid excretion was significantly higher on diets containing 64% casein than on diets containing 24% casein and paralleled the increased growth and survival observed at the higher protein level. Urinary excretion increased with age on the 64% casein diet.

2. Fecal pantothenic acid excretion was higher when rats were fed than when they were fasted and was notable for its variability. During the first 90 days there was no significant difference in fecal excretion, regardless of the casein level. With increasing age and body weight there was a tendency for fecal output to increase on the higher protein level.

3. When intermediate levels of casein were used, growth, survival and urinary pantothenic acid (but not fecal pantothenic acid) were proportional to the casein level.

4. When low levels of pantothenic acid equivalent to that present in the high casein diets (by microbiological assay) were added to the 24% casein diet, growth and urinary pantothenic acid were not equal to those observed on the higher casein diets. When approximately double the equivalent amount of pantothenic acid was added to the 24% casein diets, the results were equivalent to those observed at the 44% casein level but were still not equal to those at the 64% casein level.

Neither increased intestinal synthesis of pantothenic acid as measured by fecal excretion nor a decreased vitamin loss by urinary excretion are responsible for the sparing action

of high casein diets on the pantothenic acid requirement of the rat. The pantothenic acid content of the casein used in the purified diet while exercising an effect can not fully account for the protective action of such diets.

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# THE EFFECT OF MIXED TOCOPHEROLS ON THE UTILIZATION OF VITAMIN A IN THE RAT<sup>1</sup>

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The relation of tocopherols or vitamin E to vitamin A utilization was first reported by Moore ('40) after he observed that a lack of adequate vitamin E in the diet of rats receiving vitamin A supplements was associated with low reserves of vitamin A in the liver. Results of studies carried out the following year in cooperation with Davies (Davies and Moore, '41) showed that in the livers of rats maintained on a diet deficient in both vitamins A and E, the vitamin A store disappeared much more rapidly than did that from livers of animals receiving dl- $\alpha$ -tocopherols. It was pointed out by these authors that the tocopherols had been used in vitro for stabilizing solutions of vitamin A in fats and that these substances might possibly exert a similar effect in vivo. The inter-relationship of vitamins A and E was subsequently studied by Bacharach ('40), Sherman ('41), Quackenbush et al. ('42), Hickman et al. ('42, '44), Guggenheim ('44), Harris et al. ('44), Popper and Volk ('44), Gridgeman ('44), Sanders et al. ('44), Dattatreya Rao ('45) and Kemmerer et al. ('47).

The object of the work presented in this paper was to investigate further the covitamin activity of tocopherols as it

<sup>1</sup> This material was presented at the American Chemical Society Meeting, September, 1946, at Chicago, Ill.

is reflected in increased growth and in the storage of vitamin A in the livers of rats. These experiments have been designed to test previous findings reported concerning vitamins A-E synergy and to gain additional information regarding the mode of action of vitamin E. More specifically the objectives of this study are: (1) to determine the effect of tocopherols on the growth response to vitamin A; (2) to investigate the influence of the tocopherol content of the diet on the growth obtained with the U.S.P. bioassay procedure for vitamin A; (3) to determine whether increasing amounts of vitamin A will compensate for a lack of vitamin E; (4) to observe the covitamin activity of tocopherols administered with vitamin A at different intervals of time and by various methods; (5) to investigate the effect of tocopherols on the storage of vitamin A in the liver of the rat when given in combination with vitamin A to animals subsisting on a vitamin A-E deficient diet.

#### PROCEDURE

The general procedure involved in these experiments is outlined below and all deviations from this procedure will be discussed in detail in separate sections under "Results."

In order to observe the effect of tocopherols on vitamin A it was first necessary to maintain the experimental animals on diets deficient in both vitamins until the animals showed signs of vitamin A deficiency as manifested by declining weight. The standard U.S.P. diet for vitamin A bioassay was used with the following modifications: (1) Jones and Foster ('42) salt mixture was substituted for that described in the Pharmacopoeia; (2) 6 U.S.P. units of vitamin D (irradiated ergosterol) per gram of ration were added and (3) the yeast and starch were washed with acetone. The type of vegetable oil was varied; diet 1 contained olive oil, diet 2 cottonseed oil and diet 3 corn oil. The tocopherols were eliminated from the diet by substituting olive oil for cottonseed or corn oil. To be certain that the ration containing olive oil was deficient in vitamin E, groups of males and females were raised to

naturity on this diet and mated. No litters were born, whereas, a comparable group of rats raised on a similar ration, supplemented with 0.3 mg daily of mixed tocopherols cast and reared normal litters. The same lot of olive oil was used throughout the experiment.

Weanling rats, distributed approximately in equal numbers according to sex (unless otherwise stated), age, and weight, were maintained on diet 1 in air conditioned quarters (75°F., 40% relative humidity). When their weights remained stationary or declined for a period of 7 successive days the rats were considered to be depleted of their vitamin A reserves. This required from 25 to 35 days. The rats were then divided into experimental groups and treated with various supplements for a 28-day test period. The oil diluent employed in administering the vitamin A and mixed tocopherols was the same as that used in the ration; either olive, cottonseed or corn oil. The supplements were made fresh each week and stored under CO<sub>2</sub> in the refrigerator when not in use.

It has been reported that the vitamin E content of oils varies widely (Sherman, '41; Dattatreya Rao, '45) and in consequence an effort was made to ascertain the effect of this variation on growth. Whenever a vitamin A supplement was used, it was either the U.S.P. Reference Oil II or a vitamin A distillate<sup>2</sup> (no. 78794) diluted in olive oil. The vitamin E supplement was a mixture of natural tocopherols.<sup>2</sup> With 2 exceptions all vitamin E supplements were given in 1 of the vegetable oils and administered orally. In 1 experiment described in Section I, the tocopherols were first dissolved in cottonseed oil and then incorporated in the diet. In another experiment where the vitamin was administered by injection (Section III) the tocopherols were solubilized with "Tween 20"<sup>3</sup> and given in an aqueous solution.

<sup>2</sup> Obtained from Distillation Products, Rochester, N. Y.

<sup>3</sup> Obtained from Atlas Powder Company, Wilmington, Delaware. Twelve gm of "Tween 20" were mixed with 970 mg of a 31% solution of natural mixed tocopherols concentrate and made to a volume of 100 ml with distilled water.

## RESULTS

*I. The supplementary action of tocopherols when administered to vitamin A depleted rats receiving the U.S.P. Reference cod liver oil*

The animals receiving diet I (table 1) without the addition of tocopherols, made an average increase in weight of 16 gm as compared with the 29 gm for a comparable group receiving the tocopherol supplement. This indicates that under the conditions of the experiment, tocopherols were necessary for optimum utilization of vitamin A. It was also noted that variable responses were obtained when the cottonseed oil diet (diet II) was used. The data show that the supplementing

TABLE 1

*The supplementary effect of tocopherols added to olive, cottonseed and corn oils on the growth response of vitamin A depleted rats receiving U.S.P. Reference cod liver oil.*

GROUP NO.	NO RATS	DIET NO	TYPE OF VEGETABLE OIL	TOCOPHEROL SUPPLEMENT <sup>1</sup>	REFERENCE COD LIVER OIL	WEIGHT GAIN IN 28 DAYS
				mg/rat/day	units/day	gm
1	12	I	Olive oil	....	2.04	16 ± 3.9 <sup>2</sup>
2	14	I	Olive oil	0.025	2.04	29 ± 4.0
3	17	II	Cottonseed oil lot I <sup>2</sup>	....	2.04	34 ± 5.5
4	13	II	Cottonseed oil lot I <sup>2</sup>	0.025	2.04	35 ± 3.9
5	22	II	Cottonseed oil lot II <sup>2</sup>	....	2.04	34 ± 3.2
6	16	II	Cottonseed oil lot II <sup>2</sup>	0.3 (per 10 grams ration)	2.04	46 ± 3.6
7	21	III	Corn oil	....	2.04	41 ± 3.4
8	20	III	Corn oil	0.3	2.04	44 ± 3.3

<sup>1</sup> Administered in terms of mixed tocopherol content.

<sup>2</sup> Two different lots of cottonseed oil were used in these studies.

<sup>3</sup> Standard error.

of diet II with 0.025 mg per day of tocopherols failed to increase the growth promoted by the vitamin A supplement although the addition of 0.3 mg of the vitamin per 10 gm of a similar ration containing another lot (lot II) of cottonseed oil did measurably increase the weight response. From the similarity in growth responses of the animals receiving the ration unsupplemented with tocopherol in which 2 different lots of cottonseed oil were used (groups 3 and 5) it seems probable that the 2 samples of oil contained equal amounts of tocopherols, although the amount available was inadequate. If the data at hand justify a comparison of the 2 lots of oil, it may be postulated that the 0.025 mg supplement of tocopherol was insufficient and that 0.3 mg were required for a further increase in growth. It should be pointed out that tocopherols incorporated in the ration (group 6) measurably increased the response to vitamin A although Hickman and associates ('42, '44) have stated that the synergistic effect of vitamin E is more significant when fed separately.

When the corn oil diet was used, the addition of tocopherols had little effect on the response to vitamin A indicating that the corn oil contained an adequate amount of tocopherols.

These data show that there is sufficient variation in the tocopherol content of different types of vegetable oils to affect the rate of growth of test animals during the usual vitamin A assay and therefore this factor should be considered in the standard U.S.P. vitamin A assay. The data confirm recent evidence that dietary supplementation with an adequate amount of tocopherol is necessary in order to secure maximum weight response to a given dose of vitamin A (Hickman et al., '44; Sanders et al., '44; Dattatreya Rao, '45).

## *II. The effect of tocopherols on the growth response of vitamin A depleted rats when given increasing amounts of vitamin A*

Prior to the 28-day treatment period all rats were maintained on diet I containing olive oil until depleted of vitamin A. The diet was unchanged during the subsequent growth test



except in the case of animals receiving daily oral supplements as indicated in table 2. One series of test animals was fed tocopherols orally at a level of 0.3 mg per day, the supplement being in combination with increasing amounts of vitamin A. The rats of the second series received comparable amounts of the vitamin A supplement without added tocopherol. The data show that the presence of tocopherols increased the growth response of rats to vitamin A within certain limits. These data also show that as the vitamin A intake was raised above 4.08 units per day, the covitamin E activity was no longer

TABLE 2

*The effect of tocopherols on the growth response of vitamin A depleted rats, when given increasing amounts of vitamin A.*

SUPPLEMENT VITAMIN A  units/day <sup>1</sup>	WITH TOCOPHEROLS 0.3 mg/DAY/RAT		WITHOUT TOCOPHEROLS	
	No. rats	Weight response in 28 days  gm	No. rats	Weight response in 28 days  gm
0	11	— 9	17	— 16
2.04	19	+ 43 ± 3.1 <sup>2</sup>	17	+ 32 ± 5.6 <sup>2</sup>
4.08	20	+ 66 ± 1.6	21	+ 54 ± 2.5
8.16	20	+ 79 ± 5.9	21	+ 84 ± 4.5
16.32	20	+ 86 ± 4.1	21	+ 87 ± 5.8
32.64	19	+ 94 ± 6.0	22	+ 97 ± 7.0

<sup>1</sup> The supplements were diluted in olive oil.

<sup>2</sup> Standard error.

apparent. If it is assumed that tocopherols have a similar effect on carotene and vitamin A, then these data indirectly support the statement of Harris, Kaley and Hickman ('44) that "small quantities of tocopherol are synergistic with small and moderate intakes of carotene. . . . There is a definite ratio between carotene and tocopherol intakes; so that the potency of larger quantities of carotene benefits from increased quantities of tocopherol." In order to show optimum utilization of large amounts of vitamin A it may be necessary to raise the level of vitamin E or use some criterion other than growth.

*III. The effect of tocopherols and vitamin A administered at various time intervals to vitamin A depleted rats*

A state of vitamin A depletion was produced in all animals by feeding the U.S.P. diet containing olive oil (diet I). The test animals were then divided into experimental groups and treated for a 28-day period with equal doses of tocopherols and similar levels of vitamin A administered as indicated in

TABLE 3

*The synergistic effect of tocopherols and vitamin A administered at various time intervals to vitamin A depleted rats.*

GROUP NO.	NO. RATS	TIMES TREATED PER WEEK	SUPPLEMENTS <sup>1</sup>		WEIGHT GAIN OR LOSS IN 28 DAYS
			Tocopherols	Vitamin A	
					<i>gm</i>
1	35	6	None	Daily — Oral	+ 21 ± 3.0 <sup>2</sup>
2	20	6	Daily Oral	None	— 8.5
3	37	6	Daily Oral	Daily — Oral	+ 48 ± 2.8
4	18	6	Daily Oral A.M.	Daily — Oral P.M.	+ 43 ± 3.1
5	18	3	Mon., Wed., Fri., Oral	Tues., Thurs., Sat., Oral	+ 39 ± 3.3
6	19	2	Wed., Sat., Oral	Wed., Sat., Oral	+ 37 ± 4.5
7	19	2	Wed., Sat., Oral	Mon., Thurs., Oral	+ 37 ± 3.7
8	57	6	Daily — Injected (Solubilized tocopherol used)	Daily — Oral	+ 40 ± 2.4

<sup>1</sup> Supplements — tocopherols — 1.8 mg per week; vitamin A — 12.24 units per week. This was equivalent to 0.3 mg tocopherols daily and 2.04 units of vitamin A daily.

<sup>2</sup> Standard error.

table 3. With the exception of the aqueous tocopherol used for injection (group 8) all supplements were diluted in olive oil and given orally.

Two control groups (1 and 2) were included, the animals of 1 group receiving vitamin A only, while those of the other group received the tocopherols alone. It may be observed that vitamin A promoted some growth in the test animals while the animals which were given tocopherols following complete deprivation of vitamin A lost weight. The animals of group 3

which served as a positive control or as a standard of comparison received orally each day both vitamin supplements dissolved together in olive oil. As would be expected these animals responded with a maximum gain in weight.

The mode of treatment was modified for the animals of groups 4, 5, 6 and 7 in that although they received the same amount of vitamins A and E per week, the interval of administration was varied. From the results obtained it appears that tocopherols given separately exerted slightly less synergistic activity than when given in combination with vitamin A. It should be pointed out, however, that the difference involved was too slight to be truly significant. Hickman et al. ('42, '44) reported that the synergistic effect of vitamin A and E was definitely reduced when they were fed on alternate days rather than simultaneously but the observations of these authors were made while using somewhat smaller doses of the vitamins.

Daily parenteral injection of an aqueous solution of tocopherols with oral administration of vitamin A was employed with animals of group 8. The average weight responses of the animals receiving this treatment were slightly lower than optimum but not significantly different from the average gain of the groups given both supplements orally.

The conditions of these experiments have been designed to gain additional information concerning the site and mechanism of covitamin E activity. Both Quackenbush et al. ('42) and Hickman et al. ('44) have postulated that the action of tocopherol is a chemical one probably occurring in the gastrointestinal tract. Hickman based his conclusion on the reduced synergism he observed when the vitamin and covitamin are given at different times or by different routes. The present data fail to corroborate these observations, however, and the response noted when tocopherol was injected parenterally suggests that its supplementary action on vitamin A is not confined to the digestive tract. A publication by Popper and Volk ('44), in which it was reported that fluorescent microscopic examination of the rat intestine showed no increased

absorption of vitamin A after the administration of tocopherols, lends indirect support to our findings. It appears that the covitamin activity of tocopherol is more extensive in scope than has been generally assumed and is not a simple chemical reaction limited to the digestive tract.

#### *IV. The effect of tocopherols on the storage of vitamin A in the liver of the rat*

With the exception of 2 groups of male rats (groups 15 and 16) which had been included for the purpose of comparison, females were used exclusively for the studies on liver storage of vitamin A. All experimental animals were provided with diet I, the U.S.P. vitamin A-free diet containing olive oil, until depleted of vitamin A. The test animals were then divided into groups and the animals of the respective groups were treated as indicated in table 4. In this treatment the same diet was continued but a daily supplement of vitamin A distillate in olive oil alone or in combination with tocopherols in olive oil was given orally. The daily allowance of tocopherols was maintained at 0.3 mg but the level of vitamin A ranged from 63 to 4,000 U.S.P. units. The animals comprising groups 1-12 were treated for 3 days with daily supplements of the vitamin A distillate administered alone to 1 group and in combination with tocopherols to a comparable group, the vitamin A supplement being increased with each succeeding pair of animal groups. After 3 days of vitamin supplementation the animals were rested for 1 day and on the fifth day were killed by decapitation. The livers from each group of rats were weighed, pooled, homogenized in a Waring blender and samples taken for the determination of vitamin A. The method used for the estimation of vitamin A in liver tissue has been outlined in a previous publication (Lemley, Brown, Bird and Emmett, '47).

The results presented in table 4 indicate that the effect of tocopherols on the storage of vitamin A in the liver of the rat cannot be demonstrated when a short term supplementing period is employed. In some instances the animals receiving

tocopherols had slightly larger liver stores of vitamin A than those treated with vitamin A alone. For example, those animals which had received vitamin A supplements of 4,000 units daily stored 4,150 units of vitamin A, while those receiving tocopherols in addition to this vitamin A supplement were found to have an average of 4,480 units in the liver. These

TABLE 4

*The effect of tocopherol intake on the storage of vitamin A in the liver of the rat.*

GROUP NO	NO RATS	SEX	TREATMENT			LIVER STORAGE OF VIT A I U of A/ Total liver	% VIT A STOPPED
			Duration of treatment	Tocopherol supplement	Vitamin A supplement		
				mg/day	units/day		
1	4	F	Three days	0.0	63	0	0
2	5	F	Three days	0.3	63	0	0
3	6	F	Three days	0.0	250	301	40.0
4	6	F	Three days	0.3	250	245	32.7
5	7	F	Three days	0.0	500	525	35.0
6	7	F	Three days	0.3	500	520	34.8
7	8	F	Three days	0.0	1000	960	32.7
8	5	F	Three days	0.3	1000	1032	31.4
9	8	F	Three days	0.0	2000	1835	30.6
10	5	F	Three days	0.3	2000	2360	30.4
11	8	F	Three days	0.0	4000	4150	34.6
12	3	F	Three days	0.3	4000	4480	37.3
13	8	F	Three months	0.0	100	718	10.0
14	8	F	Three months	0.3	100	1228	17.0
15	4	M	Three months	0.0	100	495	6.9
16	4	M	Three months	0.3	100	1360	18.9
17	4	F	Six months	0.0	100	2270	15.8
18	10	F	Six months	0.3	100	3400	23.6
19	11	F	Six months	0.0	200	6988	24.3
20	10	F	Six months	0.3	200	9025	31.3

differences are not considered significant, however, since it has been reported that large variations in liver reserves of vitamin A are found in similar animals receiving identical treatments (Bacharach, '40).

It should be pointed out that in these studies large doses of vitamin A were employed in order to produce appreciable liver storage in a short time and that any increase in the

liver content of vitamin A due to the addition of tocopherols would be relatively slight. The data in table 4 indicate that there is no marked benefit resulting from taking vitamin A and E together at doses sufficient to cause vitamin A liver storage although there does appear to be a trend toward higher liver stores when tocopherols are given with daily doses of 1,000 to 4,000 units per day of vitamin A. But, as has been mentioned above, these differences are not significant.

Guggenheim ('44) has recently reported that the daily administration of vitamin E in combination with 100 units of vitamin A on 2 successive days increased the liver storage of vitamin A. After the vitamin A was given alone he found 18.5 units per gm of liver tissue but when the same amount of vitamin A was fed with 0.1 to 10 mg of tocopherols, the liver reserves of vitamin A ranged from 22 to 45 units per gm of tissue. In the experiments of this author lower doses of vitamin A were used and the effect of tocopherols became apparent, although our results indicated that tocopherols given with high doses of vitamin A had no noticeable effect on liver storage.

In the later experiments a longer treating period, either 3 or 6 months, was adopted. After the animals had been depleted of vitamin A they were maintained on diet I supplemented as indicated in table 4. In order to be certain that vitamin E was not present in the basal diet, breeding records were also kept on representative females from groups 13, 14, 15 and 16. Six of the females in group 13 which were being fed an olive oil diet unsupplemented with tocopherols became pregnant but no litters were cast, thus indicating that resorption had occurred owing to a lack of vitamin E. Six of the 8 females in group 14 receiving daily supplements of tocopherols bore normal litters. The breeding records of the animals in groups 13 and 15 definitely established the deficiency of vitamin E in the diet. After the rats had undergone treatment for 3 months they were killed and the livers were assayed for vitamin A, the livers from the 2 groups of males

(groups 15 and 16) being similarly assayed. It was thought that pregnancy and lactation might affect the vitamin A storage ability of the females but when their liver reserves were determined and compared with those of males, similar vitamin values were noted. In consequence it was assumed that although these animals had previously been used for breeding experiments the storage of vitamin A in their livers was a reliable indication of the effect of tocopherol treatment. From the results reported in table 4 it can be seen that the females depleted of tocopherols (group 13) stored an average of 718 units of vitamin A while the females of group 14, which had received the tocopherols, stored 1,228 units and likewise the addition of tocopherols to the diet of male rats raised the liver content of vitamin A from 495 to 1,360 units. It was, therefore, concluded that the daily administration of 0.3 mg of tocopherols over an extended period (3 months) increased the liver storage resulting from a daily intake of 100 units of vitamin A.

The storage of vitamin A in the livers of the rats was also determined after feeding a daily supplement of 100 or 200 units of vitamin A alone or combined with 0.3 mg of tocopherols for a period of 6 months. The effect of tocopherols feeding on the storage of vitamin A is evident when one compares the average liver storage of 2,270 units as reported for the animals of group 17 on vitamin E deficient diet with a storage of 3,400 units as found for the animals of group 18 which had received the tocopherol supplement. Similar results were obtained when the vitamin A intake was raised to 200 units daily. The data presented in table 4 show that animals treated for 6 months stored approximately 3 times as much vitamin A in their livers as did similar animals which had been treated for only 3 months. This increase occurred irrespective of tocopherol supplementation and the effect of tocopherols on the liver storage was somewhat less significant at the end of the 6-month feeding period than it had been at the end of the 3-month period. It may also be noted that the increase in liver storage promoted by the inclusion of

tocopherols was less when a daily level of 200 units of vitamin A was given than when the previously mentioned 100-unit daily supplement was employed. In other words, in these experiments the favorable effect of tocopherols on the storage of vitamin A in the liver of the rat was diminished by extending the supplementing period and by raising the daily intake of vitamin A. It is probable that the covitamin activity of tocopherols would have been more pronounced if smaller quantities of vitamin A had been employed, as Hickman et al. ('44) have stated that the A-E synergism is more active at lower doses of vitamin A.

These experiments confirm the previous reports of Moore ('40) and Bacharach ('40) that tocopherols increase the storage of vitamin A in the liver of the rat when treated for long periods; however, we have been unable to demonstrate any effect of tocopherols when the rats are treated with vitamin A for only 3 days.

#### SUMMARY

1. Growth studies indicated that when vitamin A was fed at a daily level of 2.04 units to rats receiving a vitamin A-E free diet, the addition of tocopherols caused a further increase in weight.

2. Variable growth responses to comparable supplements of vitamin A were obtained when different types of vegetable oil were included in the U.S.P. vitamin A free diet. When the U.S.P. diet containing either olive or cottonseed oil was used, daily feeding of 0.3 mg of tocopherols increased the weight gain promoted by a vitamin A supplement but the addition of tocopherols to a corn oil diet had no effect on the growth of rats receiving vitamin A.

3. The supplementary effect of tocopherol administration on growth was apparent when vitamin A was fed at low levels but this effect gradually disappeared with an increasing intake of vitamin A.

4. Tocopherols exerted a supplementary effect on vitamin A utilization whether the vitamin A and tocopherols were given



together or on separate days. A water soluble preparation of tocopherols administered by injection was also shown to increase the growth response to vitamin A.

5. The storage of vitamin A in the liver of the rat was increased when tocopherols were given with vitamin A for an extended period.

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# METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

## XIII. THE UTILIZATION OF NIACIN DURING LACTATION<sup>1</sup>

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Following the discovery by Elvehjem, Madden, Strong and Woolley ('38) that niacin could cure canine blacktongue, the counterpart of human pellagra, this vitamin has been used extensively to treat pellagra and associated deficiency states in human beings. Since niacinamide is a component of coenzymes I and II, it has been generally assumed that its metabolism is closely associated with tissue respiratory mechanisms involving these pyridine nucleotides.

Hamilton and Hogan ('44) found that niacin and choline were necessary for successful lactation in the hamster but little has been done to test any possibility of a relationship between niacin metabolism and lactation. In efforts to expand available knowledge of the composition of human milk and the metabolism of women during the reproductive cycle, we have determined the amounts of niacin in the 24-hour intakes of food, secretions of milk and excretions of urine by healthy mothers who were successfully nursing their infants.

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## PROCEDURE

Niacin intake, secretion in milk and excretion in urine were studied with 7 healthy nursing mothers during the first 10 days postpartum and with 9 women during periods of 5 consecutive days 2 to 10 months postpartum. Earlier papers have presented the organization of the study (Macy, Williams, Pratt and Hamil, '45) the method of manual expression used in collecting the complete 24-hour secretions of milk (Davies, '45), the diets and preparations of the food and milk (Kaucher, Moyer, Richards, Williams, Wertz and Macy, '45), urine (Roderuck, Williams and Macy, '46), and the concentration of niacin in immature and mature human milk (Coryell, Harris, Miller, Williams and Macy, '45).

## METHODS

The preparation of the milk samples for assay and the microbiological procedure employing *Lactobacillus arabinosus* have been described (Coryell et al., '45).

In each 5-day period of study all subjects received diets comparable in food distribution but adjusted in level so that the appetites of the women were satisfied. Aliquots of each serving of every food eaten during a 5-day period were collected, combined, ground and thoroughly mixed. For the analyses for niacin, pantothenic acid and biotin,<sup>3</sup> a 400-gm quantity of the food composite was homogenized in a Waring Blendor, transferred quantitatively to a 500-ml volumetric flask and diluted to volume. Duplicate aliquots (5 ml) were pipetted into 250 ml Erlenmeyer flasks, 50 ml of 2 *N* sulfuric acid added to each flask and the mixtures autoclaved for 3 hours at 15 pounds pressure. After cooling, the contents were brought to pH 5.0 with sodium hydroxide, diluted to 500 ml with distilled water, and filtered through Whatman no. 42 paper. The dilution of the filtrate was made 1:200 or 1:250 before assay. Quantities of 1, 2, 3, 4 and 5 ml of the diluted filtrates, in duplicate, were placed in 10 assay tubes

<sup>3</sup> Papers presenting the results for biotin and pantothenic acid are being prepared.

and standard amounts of basal medium added. After autoclaving the tubes were inoculated, incubated, and the growths measured turbidimetrically and titrimetrically as described for milk.

Control experiments with the food composites showed that hydrolysis with hydrochloric or sulfuric acids of 1 *N* or more gave maximum niacin values, that is, values equivalent to those obtained with alkali hydrolysis. Krehl and Strong ('44) and Krehl, Elvehjem and Strong ('44) have shown the presence of a naturally occurring precursor of niacin in certain biological materials, notably cereals, which is apparently utilized by the animal organism but not by *L. arabinosus*. However, the question of its complete utilization by the animal organism is controversial. This precursor, presumably an alkyl ester of niacin, is labile to alkali or strong acids but not appreciably to weak acids (0.1 *N*). With the food composites analyzed, autoclaving with 0.1 *N* hydrochloric acid for 30 minutes gave values which averaged 9% lower than those obtained after autoclaving with 1 *N* or stronger acids for the same time. Since the use of 2 *N* sulfuric acid for 3 hours was found to be optimum for the release of bound biotin<sup>4</sup> and gave values for niacin identical with those obtained using 1 *N* acid for 30 minutes, the hydrolysis with stronger acid was used in the determination of both vitamins. Sodium hydroxide was employed to neutralize the sulfuric acid, since the values were found not to differ from those obtained using barium hydroxide.

Aliquots of the 24-hour collections of urine were filtered and the pH adjusted to 6.5 to 7.0 then diluted to suitable concentrations, usually 1:20, and assayed by the microbiological procedure described for milk. The values obtained by this method represent niacin, niacinamide, and any nicotinuric acid present. As with the milk, no attempt was made to differentiate between these 3 metabolites in the urine.

<sup>4</sup> See footnote 3, p. 220.

Knowledge concerning the fluorescent nicotinic acid metabolite in the urine, noted in 1940 by Najjar and Wood and in 1943 designated as  $F_2$ , was limited until Huff and Perlzweig ('43a) isolated N<sup>1</sup>-methylnicotinamide chloride<sup>5</sup> from urine and identified it as " $F_2$ " or its immediate precursor. Semi-quantitative values for N<sup>1</sup>-methylnicotinamide in urine were obtained for 14 5-day periods by a method similar to that of Huff and Perlzweig ('43b), in conjunction with thiamine determinations which have been published (Roderuck, Williams and Macy, '45, '46). The blank employed was the sulfite blank of Mason and Williams ('42). The eluate was treated with alkaline ferrieyanide according to the thiochrome procedure. According to Najjar and Ketron ('44) the sulfite destroys some " $F_2$ " and the ferrieyanide converts " $F_2$ " (a pyridine derivative) into pyridone, which possesses only 21% of the original fluorescence. Control experiments showed the destruction of N<sup>1</sup>-methylnicotinamide by the sulfite to be rather variable, averaging 23%. The decrease in fluorescence as a result of ferrieyanide treatment was uniform, averaging 76%, whether standard solutions of N<sup>1</sup>-methylnicotinamide or urine samples previously treated with sulfite were employed. If an average of 23% of the N<sup>1</sup>-methylnicotinamide in the urine was destroyed by the sulfite treatment and of the remaining 77% only 24% of the resulting fluorescence remained after ferrieyanide treatment, the values obtained for N<sup>1</sup>-methylnicotinamide in the urine, using the sulfite, are 19% lower than with those which would be obtained using the more commonly employed water blank. Since this work was completed a more reliable method of analysis has been published by Huff and Perlzweig ('47).

#### RESULTS AND DISCUSSION

The volumes of milk and urine collected each 24 hours during the first 10 days postpartum and their niacin contents are given in table 1. Values for N<sup>1</sup>-methylnicotinamide in

<sup>5</sup> The N<sup>1</sup>-methylnicotinamide chloride against which the quinine sulfate solutions were standardized was provided by Dr. W. A. Perlzweig, Duke University Medical School, Durham, N. C.

TABLE 1

*Niacin excretion in urine and secretion in milk during first 10 days postpartum*

INTERVAL POSTPARTUM	VOLUMES		NIACIN		N <sup>1</sup> -METHYL- NICOTINAMIDE CHLORIDE	INTERVAL POST- PARTUM	VOLUMES	
	Milk	Urine	Milk	Urine			Milk	Urine
days	ml	ml	mg	mg	mg	days	ml	ml
Subject: V.L.						Subject: V.K.		
1	30	1757	0.04	1.23	1.4	1	9	3086
2	56	2686	0.04	1.26	..	2	90	3651
3	353	2759	0.22	1.33	1.6	3	484	2596
4	794	1823	0.41	1.48	1.2	4	547	2427
5	844	1847	0.50	1.41	1.8	5	560	2331
6	955	2710	0.64	1.21	2.6	6	663	2249
7	1047	2841	0.83	1.45	1.8	7	781	3380
8	1098	2066	1.24	1.15	4.1	8	775	3840
9	1118	2151	1.53	1.64	2.8	9	794	3115
10	1200	1669	2.06	1.47	1.6	10	797	3328
Subject: V.S.						Subject: J.M.		
1	6	1869		0.97	1.7	1	35	2182
2	92	1874	0.07	0.88	1.1	2	385	1386
3	420	2887	0.24	0.86	3.7	3	870	1519
4	600	2063	0.38	0.83	1.9	4	1011	1201
5	697	2065	0.51	0.92	4.5	5	1121	1283
6	756	1502	0.77	0.67	3.1	6	1125	1172
7	818	2524	1.25	0.92	2.0	7	1287	1161
8	837	2237	1.82	0.73	2.1	8	1136	1520
9	932	1984	2.38	0.78	2.7	9	1258	....
10	924	2287	2.78	0.68	1.7	10	1336	1553
Subject: L.F.						Subject: C.O.		
1	71	2590	0.05	1.32		1	16	1864
2	200	1275	0.12	0.94		2	100	939
3	733	2420	0.66	1.31		3	335	1511
4	1122	2075	0.85	1.12		4	595	....
5	1441	1674	1.21	1.12		5	725	....
6	1501	1718	1.56	1.23		6	821	....
7	1596	1340	2.42	1.18		7	798	1470
8	1638	1810	3.44	1.25		8	950	1127
9	1676	1374	4.47	0.44		9	931	1191
10	1872	2220	5.69	1.31		10	660	....
Subject: V.G.						Subject: M.		
1	82	2067	0.07	0.69		9	1189	1035
2	177	1689	0.11	0.72		Subject: E.		
3	849	1775	0.49	0.78		8	688	677
4	1413	1323	0.82	0.66		Subject: M.		
5	1471	1303	0.81	0.83		7	880	1355
6	1782	1148	1.07	0.71		Subject: G.		
7	1630	1230	1.28	0.71		7	1017	1981
8	1895	1237	2.02	0.68		Subject: F.		
9	1828	1104	2.56	0.61		7	953	1032
10	1770	1075	2.79	0.63				

<sup>1</sup> The first day postpartum was variable to the extent of differences in the times at which the women delivered. For subjects delivered after 12 M. the first day began the following morning. The first day for C.O. was 22 hours; for L.F. 23.5 hours. V.G. was delivered 8 hours before the beginning of the first day postpartum; V.K. 17 hours; V.L. 5 hours; J.M. 16 hours; and V.S. 7 hours.

the urine of 2 women during the puerperium also are given in table 1. For each 5-day period of study table 2 presents the average daily energy intake, milk and urine volumes, and the amounts of niacin in food intake, in the milk and in the urine. The amounts of N<sup>1</sup>-methylnicotinamide determined in urine during certain periods are also included in table 2. For intake of niacin both the values obtained by hydrolysis with weak acid, and those for niacin plus its precursor, obtained by hydrolysis with strong acid, are given in the table.

The food eaten by the mothers during the first 10 days postpartum provided averages for the 5-day periods of 13 to 22 mg of niacin and its precursor per day. The rapid increases in the amounts of niacin secreted daily in milk portray both the increases in concentration (Coryell, Harris, Miller, Williams and Macy, '45) and volume during the puerperium. Niacin in the urine varied from day to day for the individual women but each subject seemed to have a characteristic level of excretion. During the puerperium neither secretion in milk nor excretion in urine show relationship with intake; nor do the daily amounts of niacin in urine show a relationship to the volumes of urine or milk, or the quantity of the vitamin in the milk.

For all of the women studied, during the puerperium and while they were secreting mature milk, the average daily intakes of niacin and its precursor during the 5-day periods ranged from 13.0 to 23.4 mg as determined for the food eaten, and the energy intakes ranged from 2256 to 3559 cal. per day, thus the niacin intakes per 1000 cal. ranged from 5.0 to 7.8 mg. The values for niacin in milk (table 2) show a general relationship to milk volume rather than to intake and illustrate the wide range of normal variation in the composition of human milk from different mothers and from the same mother at different times. Greater amounts of niacin were excreted in the urine during the 2 5-day periods immediately postpartum than during periods later in lactation.

The percentage of the average daily niacin intakes secreted in milk ranged from 1 to 3 for the first 5 days postpartum,

TABLE 2

*Average daily niacin intakes, excretion in urine and secretion in milk during 51 5-day study periods.*

INTERVAL POST- PARTUM	ENERGY INTAKE	VOLUMES		NIACIN								N <sup>15</sup> - METHYL- NICO- TINAMIDE CHLORIDE
		Milk	Urine	Intake		Milk		Urine		Urine		
				Total <sup>1</sup>	Free							
	days	cal.	ml	ml	mg	mg	mg	% intake	mg	% intake	mg	
B.	72-76	2547	718	691	14.8	13.4	1.34	9	0.35	2		
C.	1-5	2850	713	2007	18.5	16.5	0.58	3	1.16	6		
	6-10	2898	1657	1692	16.1	14.1	3.52	22	1.08	7		
D.	1-5	2475	798	1631	13.6	12.4	0.46	3	0.74	5		
	6-10	2445	1781	1159	13.2	11.8	1.95	15	0.67	5		
	78-82		848	1281	23.4	20.9	2.02	9	0.69	3		
	161-165	2873	901	993	19.4	17.1	1.83	9	0.50	2	0.9	
	239-243	2925	681	810	17.0	15.7	1.16	7	0.48	3	4.4	
	302-306 <sup>2</sup>	2827	394	1080	18.7	16.9	0.64	3	0.51	3	4.6	
E.	1-5	2403	338	2818	13.0	11.7	0.30	2	0.71	5		
	6-10	2381	762	3182	15.0	13.3	1.73	12	0.81	5		
	95-99	2822	647	2804	14.6	13.6	1.30	9	0.52	4	0.8	
	144-148 <sup>2</sup>	2806	325	1447	14.7	13.6	0.56	4	0.48	3	2.4	
F.	1-5	3167	415	2174	18.8	16.6	0.24	1	1.34	7	1.5	
	6-10	3365	1084	2287	22.6	20.8	1.26	6	1.38	6	2.6	
	68-72	3275	789	2849	21.6	19.0	1.77	8	1.08	5	4.0	
	152-156	3253	680	1756	21.2	18.6	1.17	6	0.53	2	4.0	
G.	1-5	3269	684	1514	16.4	15.4	0.49	3	0.85	5		
	6-10	3412	1228	1352	18.8	18.3	2.26	12	0.90	5		
	75-79	3225	708	2265	20.2	19.4	1.57	8	0.67	3	4.7	
	173-179 <sup>2</sup>	3559	268	2003	20.5	19.2	0.52	2	0.68	3	5.5	
H.	1-5	2256	354	1438	13.4	12.6	0.28	2	0.87	6		
	6-10	2452	832	1263	13.6	13.8	1.44	10	0.90	7		
I.	85-89	2762	1020	1347	19.1	17.6	1.71	9	0.56	3		
	204-208	2825	913	1356	18.9	16.6	2.00	10	0.63	3		
	259-263	2918	676	1219	20.6	17.8	1.40	7	0.70	3		
J.	58-62	2735	947	939	18.4	16.7	1.31	7	0.48	3		
K.	80-84	2653	899	1078	20.8	19.5	1.65	8	0.62	3		
L.	1-5	3278	363	2152	18.0	16.2	0.24	1	0.89	5	2.6	
	6-10	3474	853	2107	20.1	18.6	1.80	9	0.76	4	2.3	
	70-74 <sup>2</sup>	3350	304	1852	20.1	16.8	0.86	4	0.63	3	8.8	

<sup>1</sup> Total indicates free + precursor. Free niacin was obtained with 0.1 N HCl.

<sup>2</sup> Lactation terminating.



with 5 to 7% of the intake being excreted in urine. For the next 5 days niacin in the milk ranged from 6 to 22% of the intake but the average daily excretions in urine were comparable to those of the preceding period. During the periods of mature milk production averages of only 2 to 10% of the intakes were secreted in milk, while only 2 to 5% was found in the urine. The data for healthy women receiving good diets do not indicate a relationship between intake and secretion in milk or excretion in urine, between secretion in milk and excretion in urine, or between urine volume and niacin content. That the data represent the physiologic performance of average healthy women whose nutritional status was good before and after delivery is emphasized by the values for V.S. who throughout pregnancy and lactation, except for the intervals of study, was ingesting daily, in addition to a good diet, 6250 I.U. of vitamin A, 3.6 mg of thiamine, 2.0 mg of riboflavin, 1000 I.U. of vitamin D, 10 mg of niacin and 0.5 mg of pantothenic acid.

The low values for niacin in the urine are in agreement with the findings of most investigators that "niacin" is quite low and uniform and has little relationship with intake, at least within a rather wide range of the latter. The urine "niacin" values include niacinamide and any nicotinuric acid (a conjugate of niacin with glycine) that may be present, since *L. arabinosus* can utilize all 3 forms. Based on chemical differentiation, the presence of nicotinuric acid in human urine under normal conditions has been controversial.

Recently Johnson ('45) developed a microbiological procedure using *Leuconostoc mesenteroides* in conjunction with *L. arabinosus* to differentiate between niacin, its amide, and the glycine conjugate. Using this method he was unable to find any nicotinuric acid in the urine of normal subjects, either while they were ingesting average diets or following supplementation with 50 mg of niacinamide per day for 5 days (Johnson, Hamilton and Mitchell, '45). With daily diets estimated to contain an average of 21.1 mg of niacin, he found an average daily urinary output of 1.15 mg of niacin

and its amide. This value is comparable to the 1.1 to 1.5 mg obtained by Sarett, Huff and Perlzweig ('42) using a chemical procedure.

The values determined for N<sup>1</sup>-methylnicotinamide in the urine of the nursing mothers, averaged 3.5 mg per day. The niacin equivalent, 2.5 mg, calculated with the factor 0.713, represented 13% of the intake. The totals of the niacin in the milk and urine, and the niacin equivalents from N<sup>1</sup>-methylnicotinamide in urine accounted for approximately 23% of the intake, on the average. If the N<sup>1</sup>-methylnicotinamide values are increased by 19% for the residual N<sup>1</sup>-methylnicotinamide in the sulfite blank (see procedure) the average value would be increased to 4.3 mg, increasing the average percentage of intake niacin accounted for from 23 to 26. The 74% of the food niacin unaccounted for would strongly indicate either the complete breakdown of a large fraction of the exogenous niacin by the body or the presence in urine of end-products of niacin metabolism which have not been recognized. This explanation has been suggested by Perlzweig and Huff ('45).

Values in the literature for average N<sup>1</sup>-methylnicotinamide elimination in the urine of normal adults eating average diets vary, in niacin equivalents, from 2.3 mg (Mickelson and Erickson, '45) to 18.7 mg (Johnson, Hamilton and Mitchell, '45). The lack of agreement between different reports may be attributable in part to the methods of analysis. Nevertheless, one of the most consistent findings is the rather wide variation between individuals and between different days with the same individual.

Numerous factors besides niacin and nicotinamide intake undoubtedly affect the excretion of this metabolite. The availability of methyl groups in the body and the condition of the liver have been suggested (Perlzweig, Bernheim and Bernheim, '43; Najjar, Hall and Deal, '45), as well as body activity (Ellinger and Coulson, '44). The upper value of the range for average daily urinary N<sup>1</sup>-methylnicotinamide was that of subject V.S. at 70 to 74 days postpartum. The 10 mg

of niacin included in the vitamin supplements taken by V.S. each day except during the study periods was equal to 55% of the average amount in the food eaten during the period of investigation. The values for her excretion of N<sup>1</sup>-methyl-nicotinamide, as the chloride, were 16.65, 7.68, 5.11, 5.48 and 8.86, mg, respectively, for the 5 days. The relatively large excretion on the first day may have resulted from the supplement prior to initiation of the study. However, the values for niacin in the milk and in the urine on the first day were either equal to or less than the corresponding values for the following 4 days.

The possibility of synthesis by intestinal flora as a factor in the variable results cannot be disregarded. Ellinger and Benesch ('45) have shown a marked decrease in the excretion of nicotinamide methochloride (N<sup>1</sup>-methylnicotinamide) by the subjects after ingestion of the "sterilising" drugs, sulfaguanidine or succinyl sulfathiazole. However, ingestion by infants of 1 gm of *l*-tryptophane and by adults of 5 gm of *dl*-tryptophane has been found to produce a "prompt and marked increase in the urinary excretion of nicotinic acid derivatives, chiefly in the methylated form" (Perlzweig, Rosen, Levitas and Robinson, '47). A comprehensive study of young women by Oldham, Davis and Roberts ('46) of the intakes and excretions of certain of the B vitamins reported values for fecal niacin ranging from 0.9 to 1.7 mg per day.

The data emphasize that greater understanding of the dietary requirements and metabolism of pregnant and lactating women is contingent upon obtaining much clearer and more complete knowledge of the physiology of milk production and secretion. As the values from our investigations are brought together, impressive evidence of the unique physiology of lactation accumulates. Many factors are inter-related in producing the over-all body changes consequent to lactation and other reports containing data for the same group of healthy nursing mothers have demonstrated that for vitamin C (Munks, Kaucher, Moyer, Harris and Macy, '47), thiamine (Roderuck, Williams and Macy, '46), ribo-

flavin (Roderuck, Coryell, Williams and Macy, '46), and vitamin A (Leshner, Brody, Williams and Macy, '47), body performance during lactation cannot be interpreted in terms of results obtained with non-pregnant, non-lactating subjects.

# SUMMARY

The niacin intake for 5-day periods and output in 24-hour collections of milk and urine were determined for normal multiparas during the first 10 days postpartum and at various intervals during mature milk production. The intakes of the women were comparable qualitatively but the quantity was determined by appetite. With an average daily intake of 16.5 mg of niacin the average secretion in milk per 24 hours increased from 0.04 mg on the first day postpartum to 2.94 mg on the tenth day. Excretion in the urine ranged from 0.92 to 0.98 mg.

The average volume of mature milk secreted per day during each of 17 5-day periods ranged from 268 to 1020 ml. The average daily niacin content of the milk ranged from 0.52 to 2.02 mg. From 0.35 to 1.08 mg of niacin were excreted in urine. During 10 5-day periods the average daily urinary excretion of N<sup>1</sup>-methylnicotinamide ranged from 0.8 to 8.8 mg averaging 4.0 mg. Of the daily niacin intakes during mature milk production, averages of 7 and 3%, respectively, appeared in the milk and urine as niacin.

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# A CHICK GROWTH FACTOR IN COW MANURE

## V. RELATION TO QUANTITY AND QUALITY OF SOYBEAN OIL MEAL IN THE DIET

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It has been reported (Rubin and Bird, '46a, b) that an all-plant protein diet containing 35% of commercially heated soybean oil meal produced suboptimal growth of chickens unless supplemented with fish meal, cow manure or extracts of cow manure.

This diet was adequate in meeting the requirements for the essential amino acids according to Almquist's data ('46). It was shown to be complete in all of the chemically characterized vitamins by Whitson et al. ('45) and in the unidentified factors (Rubin and Bird, '46a), except for the growth factor which was obtained as a concentrate from extracts of cow manure (Rubin and Bird, '46b).

Whitson et al. ('46a) described the detrimental effect of high levels of soybean oil meal in the diet of hens on the hatchability of their eggs. The diets, although unusually high in protein, apparently were not toxic to the hens nor did they adversely affect egg production. It appeared that for each increment of soybean oil meal in the breeder diet, an additional increment of a substance which interfered with embryonic development was deposited in the egg. Ham and Sandstedt ('44) showed that there is a growth inhibitor in raw soybean oil meal presumably identical with the trypsin inhibitor described by them and crystallized by Kunitz ('46).

<sup>1</sup> Resigned.



They also indicated that the growth inhibitor is heat labile. Therefore, it would probably be destroyed in commercially heated soybean oil meal. Furthermore, the inhibitor was shown to be a protein and hence its absorption through the gut, per se, appears very unlikely. Therefore, the low hatchability reported by Whitson et al. ('46a) is probably due to some other cause than this heat labile inhibitor of trypsin and of growth.

Bird and Mattingly ('45) reported that they obtained optimum growth with soybean and corn diets which were supplemented with 0.2% methionine. Evans and McGinnis ('46) obtained improved chick growth with soybean diets supplemented with methionine. Bird et al. ('47) showed that the addition of crystalline methionine to the 35% soybean oil meal diet produced as good growth as did the growth factor of cow manure provided this growth factor had been present in the diet of the dams. Methionine was less effective when fed to the progeny of hens whose diet was deficient in the growth factor. This partial effectiveness of methionine is difficult to explain since the basal diet is supposed to contain adequate quantities of the essential amino acids. Some interrelation of the growth factor and methionine is indicated. The growth factor might conceivably facilitate the liberation of methionine from soybean protein in the digestive tract, it might function in the metabolism of methionine after absorption, or it might have some entirely different metabolic function which could be partially performed by methionine.

The literature on the value of cystine and methionine as supplements to raw and heated soybean oil meal has been reviewed by Barnes and Maack ('43). Evans and McGinnis ('46) recently reported on the availability of the sulfur amino acids of raw and autoclaved soybean oil meal alone and when supplemented with methionine. Since the sulfur-containing amino acids improve the nutritional value of either raw or heated soybean oil meal, it was of interest to determine what effect the growth factor of cow manure would have on the

nutritional value of all-plant-protein diets in which raw soybean oil meal was the major source of protein.

The objects of the experiments described in this paper were first, to determine whether the inhibition of embryonic development by high levels of commercially heated soybean oil meal would be paralleled by inhibition of the growth of chicks fed an all-plant-protein diet containing high levels of such soybean oil meal, and second, to determine the effect of the growth factor of cow manure or methionine on the growth of chicks fed high levels of heated or ordinary levels of raw soybean oil meal.

#### METHODS AND RESULTS

The chicks in all of the experiments except 6 and 7 were the progeny of crossbred dams (Rhode Island Red X Barred Plymouth Rock) and New Hampshire cocks. The chicks in experiments 6 and 7 were the progeny of Rhode Island Red dams and Barred Plymouth Rock cocks. All chicks were fed the basal diet during the first 2 weeks after hatching. At 2 weeks of age they were divided into experimental groups. The distribution into groups was made according to weight; the middle weight group was used and the light and heavy chicks were discarded. All experiments were terminated when the chickens were 6 weeks old.

The percentage composition of the basal diet was as follows: yellow corn 38.0, barley 20.0, alfalfa leaf meal 3.0, soybean oil meal 35.0, butyl fermentation solubles (containing 250  $\mu$ g of riboflavin per gm) 0.6, steamed bone meal 1.5, lime stone 1.0, salt (96% NaCl, 4%  $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$ ) 0.7, and vitamins A and D feeding oil (400 A.O.A.C. units of vitamin D, 2000 U.S.P. units of vitamin A per gm) 0.2. One mg of nicotinic acid was added to each 100 gm of this diet.

When this diet was modified to contain 25% of soybean oil meal, corn replaced 10% of the soybean oil meal of the basal diet. The diets containing 50% soybean oil meal were formulated by increasing the level of this material and decreasing the level of barley. In the 70% soybean oil meal diet,

this material replaced all of the barley and a portion of the corn. In the diets containing raw soybean oil meal or soybean flour, this material replaced the heated soybean oil meal of the basal diet. When other supplements were used, these replaced the corn of the basal diet. A sample of raw soybean oil meal was obtained from each of 2 processors, and from a third processor samples of raw and hydrolysed soybean flour were obtained.

Except in experiment 6, all the chicks were the progeny of hens which had been fed an all-plant-protein diet for several months. Therefore, they were well depleted of the growth factor of cow manure.

Experiments 1 to 5, the results of which are summarized in table 1, were undertaken to determine how well chicks would grow when fed high levels of soybean oil meal with or without supplements of the growth factor or of methionine. Experiments 1, 2 and 3, were conducted in batteries and 4 and 5 in a brooder house, the birds being brooded on the floor under electric hovers.

The results of experiment 1 show that the best growth was obtained with the 35% soybean oil meal diet. The chickens fed the 25% soybean oil meal diet did not grow quite so well. A marked inhibition of growth occurred in the group fed the 70% soybean oil meal diet in spite of the fact that the diet was supposedly complete and had an excess of essential amino acids.

Experiment 2 was designed to study the effect of the growth factor of cow manure when added to diets containing large quantities of soybean oil meal. In experiment 2, the usual stimulation in growth occurred when the basal diet containing 35% soybean oil meal was supplemented with the acid precipitate fraction of an extract of cow manure (Rubin and Bird, '46b). A depression in growth occurred when the birds were fed the diet containing 50% soybean oil meal; growth inhibition was even more marked when the diet contained 70% soybean oil meal. Remarkable results were obtained with both of these high soybean oil meal diets when they were

supplemented with the acid precipitate fraction. Growth was as good as or better than that obtained in the group fed the basal diet supplemented with the growth factor from cow manure. The results of experiment 3 confirmed the results of experiment 2.

TABLE 1

*Effect of supplements on growth of chickens fed different quantities of soybean oil meal.*

EXPERIMENT NO.	SOYBEAN OIL MEAL IN DIET	SUPPLEMENT TO DIET	AV. WT. OF CHICKENS AT 6 WKS	NO. OF CHICKS STARTED	NO. OF SURVIVORS
	%		gm		
1	25	None	378	12	11
	35	None	408	12	12
	70	None	256	12	10
2	35	None	361	12	11
	35	0.075% A.P. <sup>1</sup>	431	12	12
	50	None	295	12	8
	50	0.075% A.P.	472	12	12
	70	None	181	12	7
	70	0.075% A.P.	430	12	11
3	35	None	340	14	14
	70	None	294	14	12
	70	0.075% A.P.	406	14	14
4	35	None	422	47	47
	70	None	365	47	34
	70	0.2% dl-methionine	304	47	37
	70	0.4% dl-methionine	350	47	39
5	70	None	295	37	29
	70	None	326	37	31
	70	5% dried cow manure	467	37	33
	70	5% dried cow manure	441	37	37
	70	0.4% dl-methionine	360	37	29
	70	0.4% dl-methionine	315	37	35

<sup>1</sup> Acid precipitate of water extract of dried cow manure.

In experiment 4 the effect of methionine on the 70% soybean oil meal diet was studied. In experiment 5 certain diets used in experiment 4 were repeated with the addition of 2 groups fed the 70% soybean oil meal diet and supplemented with 5% cow manure. The results of both experiments show that

methionine had little or no counter-effect on the high soybean oil meal diet. On the other hand, the groups fed the diet containing cow manure grew at an optimum rate. The chicks used in all of these experiments were from the eggs of hens whose diet had been deficient in growth factor, and it would be expected, therefore, that the chicks would have shown less response to methionine than to the growth factor even if they had been fed these supplements with 35% of soybean oil meal (Bird et al., '47). The 70% level accentuated the difference between the 2 supplements to the extent that methionine produced little or no stimulus to growth in experiments 4 and 5.

TABLE 2

*Effect of maternal diet on chickens fed diets high in soybean oil meal.  
(Experiment 6.)*

MATERNAL DIET NO.	SOYBEAN OIL MEAL IN CHICK DIET	A.P. <sup>1</sup> IN CHICK DIET	AV. WT. AT 6 WKS.	NO. OF SURVIVORS
	%	%	gm	
311	35	0	238	11
311	35	0.075	425	16
312	35	0	383	20
312	70	0	315	16

<sup>1</sup> Same as in table 1.

Experiment 6 was designed to determine the effect of the growth factor in the maternal diet on the growth of chicks fed the high soybean oil meal diet. There were 4 groups of 20 chicks. Two groups were composed of chicks whose dams were fed a diet (diet 311, Rubin and Bird, '46b) deficient in the growth factor; the other 2 groups were composed of chicks whose dams were fed a diet (diet 312) which contained the factor. The chicks in this experiment were reared in batteries. The data are given in table 2.

In this experiment, 70% of soybean oil meal reduced the growth rate of the progeny of hens fed a "good" diet (no. 312), but these chicks, even when fed the high level of soybean oil meal, were still able, by virtue of the diet of their dams, to grow more rapidly than those fed 35% of soybean oil meal

but subject to the disadvantage of a poor maternal diet. This is evidence that the growth factor, even when stored in the chick's body, counteracted the inhibitory effect of a high soybean diet.

In all of these experiments excessive quantities of soybean oil meal in the diet caused considerable mortality which was prevented by the addition of the growth factor of cow manure.

Experiments 7 to 11, the results of which are summarized in table 3, were undertaken to determine the effectiveness of the growth factor in overcoming the deficiency of raw soybean oil meal and to compare its effectiveness with that of methionine and with the effectiveness of hydrolysis of the soybean product. The experimental groups, each consisting of 12 chicks, were reared in battery brooders, except that in experiment 8 groups of 36 chicks were brooded under electric hovers in the brooder house.

In experiment 7, the usual difference between the basal diet containing heated soybean oil meal and the same diet supplemented with the growth factor was observed. Methionine improved this basal diet but not quite so much as did the growth factor. The expected poor growth was obtained with the basal diet containing raw soybean oil meal. The growth factor improved this diet considerably but optimum growth was not achieved. Methionine in the raw soybean oil meal diet made it equal to the heated soybean oil meal basal diet.

The results of experiment 8 were essentially the same as those of the preceding one. Raw soybean oil meal no. 1 was inferior to the heated soybean oil meal. The growth factor, supplied by the dried cow manure improved the raw soybean oil meal diet considerably but did not make it equal to the diet containing heated soybean oil meal plus cow manure.

In experiment 9 a second sample of raw soybean oil meal, obtained from another processor, was compared with the first sample. This experiment confirmed the results of the first 2 experiments. About the same growth response was

obtained with the 2 different samples of raw soybean oil meal.

The second sample of raw soybean oil meal was retested in experiment 10. The results confirmed the results of the previous experiment. The growth factor improved the nutritional value of raw soybean oil meal but not to the extent obtained with heated soybean oil meal.

TABLE 3

*Effect of supplements on growth of chickens fed heated and unheated soybean products as 35% of diet.*

EXPERIMENT NO.	SOYBEAN PRODUCT IN DIET	SUPPLEMENT TO DIET	AV. WT. OF CHICKENS 6 WKS. gm
7	Heated oil meal	None	356
	Heated oil meal	0.075% A.P. <sup>1</sup>	440
	Heated oil meal	0.2% dl-methionine	390
	Raw oil meal, no. 1	None	323
	Raw oil meal, no. 1	0.075% A.P.	394
	Raw oil meal, no. 1	0.150% A.P.	414
	Raw oil meal, no. 1	0.2% dl-methionine	360
8	Heated oil meal	None	361
	Heated oil meal	5% dried cow manure	498
	Raw oil meal, no. 1	None	326
	Raw oil meal, no. 1	5% dried cow manure	429
9	Heated oil meal	None	290
	Heated oil meal	0.075% A.P.	419
	Raw oil meal, no. 1	None	247
	Raw oil meal, no. 1	0.15% A.P.	337
	Raw oil meal, no. 1	0.30% A.P.	345
	Raw oil meal, no. 2	None	244
	Raw oil meal, no. 2	0.135% A.P.	349
10	Heated oil meal	None	236
	Heated oil meal	0.075% A.P.	383
	Raw oil meal, no. 2	None	199
	Raw oil meal, no. 2	0.150% A.P.	332
	Raw oil meal, no. 2	0.225% A.P.	312
11	Heated oil meal	None	347
	Heated oil meal	0.075% A.P.	407
	Raw flour	None	263
	Flour hydrolysate	None	314

<sup>1</sup> Acid precipitate of water extract of dried cow manure.

In experiment 11, the effect of enzymatic hydrolysis of the proteins in soybean flour was studied. The flour and the hydrolysate of the flour were commercial products. Unfortunately, only enough of the hydrolysate was available for 1 experimental group; therefore, the supplementary effect of the growth factor could not be studied. The results of this experiment showed that while enzymatic hydrolysis of raw soybean flour improved it, it was not quite so good as heated soybean oil meal which was not supplemented with the growth factor. The group fed the growth factor with heated soybean oil meal was superior to all of the other groups.

#### DISCUSSION

The results of experiments 1 to 6 show that there is a parallelism between the effect of increasing levels of soybean oil meal on embryonic development (hatchability), as shown by Whitson et al. ('46a), and the effect on chick growth. Just as increasing quantities of soybean oil meal in the hen's diet caused a reduction in hatchability, increasing quantities of soybean oil meal in the chick's diet caused a reduction in viability and growth rate. The parallelism was also evident in the effect of cow manure. The addition of this material or concentrates prepared from it to diets containing high levels of soybean oil meal prevented the inhibition of growth and mortality which usually occurred among chicks fed such diets. Whitson et al. ('46b) and Bird et al. ('46) have reported that the addition of cow manure to breeder diets containing higher levels of soybean oil meal prevented the usual reduction in hatchability of eggs and viability of progeny.

The addition of methionine at levels up to 0.4% of the diet containing 70% soybean oil meal produced little or no increase in growth above that produced by the control diets. Several authors have shown that diets composed chiefly of corn and soybean oil meal can produce optimum growth when supplemented with methionine, yet methionine failed to overcome the detrimental effect of large quantities of soybean oil meal. This is evidence that the ill-effect of soybean oil meal



is not limited to interference with liberation of methionine in the digestive tract.

The fact that the growth factor of cow manure stored in the chick's body was able to counteract the effect of high soybean oil meal diets is additional evidence that the relationship involving the soybean oil meal, the growth factor and free methionine is not limited to the intestine.

It is also evident from the results of experiments 7 to 10 that the growth factor did not obviate the necessity of heat-treating soybean oil meal. Although the growth factor improved the nutritional value of raw soybean oil meal in an all-plant-protein diet, the use of heated soybean oil meal with the growth factor produced still better results.

From the evidence presented, it may be postulated that soybean oil meal contains an inhibitor of growth which is stable to heat, which produces an effect that is not confined to the digestive tract, and which, therefore, is not identical with the heat labile trypsin inhibitor reported by Ham and Sandstedt ('44) and crystallized by Kunitz ('46). The growth factor of cow manure has little or no effect upon the heat-labile inhibitor but counteracts the inhibiting effect which is not influenced by heat.

As an alternative to this hypothesis one might attempt to explain the differences in growth among groups fed 35, 50 and 70% soybean oil meal on the basis of the different quantities of barley and corn in the diets. It would be necessary to assume that these grains contain considerably more of the growth factor than does soybean meal. The report of Whitson et al. ('45) although it does not rule out this explanation completely, makes it difficult to believe that corn and barley contain significant quantities of the growth factor.

The distribution of the postulated heat-stable inhibitor in different samples of soybean products appears to be quite variable. Although no detailed study of this phase of the problem has been made, some samples of soybean oil meal have been obtained which produced optimum growth without a supplementary source of the growth factor when fed as

35% of the diet. Such meals have not been tried at the 70% level.

Further work is needed to determine if this growth inhibitor is confined to soybean products, and if the sole function of the growth factor of manure is to counteract this effect.

The results reported here would indicate that the benefits conferred by heating soybean oil meal and by supplementation with the growth factor are independent of each other. This subject should not be dismissed, however, without reference to the ability of methionine to act as a partial substitute for the growth factor (Bird et al., '47) and to the generally accepted role of this amino acid in the changes induced by heating soybean oil meal. According to Melnick et al. ('46) the poor utilization of raw soybean protein is due to inhibition of trypsin and consequent retarded liberation of methionine. Therefore, equally good results should be obtained with raw and with heated soybeans if each were supplemented with adequate methionine. Such results were not obtained either by Hayward and Hafner ('41) or by Almquist et al. ('42), perhaps because the highest levels of methionine they added to the raw soybean were inadequate or perhaps because the trypsin inhibitor disturbs protein digestion in other ways not involving the sulfur-containing amino acids. Heated soybean, because of the increased availability of its methionine, provides more of this amino acid for structural use and, if necessary, as a partial substitute for the growth factor than does the raw material. Even heated soybean oil meal, however, contains barely adequate, or slightly inadequate quantities, and so responds to methionine supplementation. It is supplemented more effectively by the growth factor.

Methionine appears, therefore, to be a connecting link between the trypsin inhibitor and the newly postulated, heat-stable growth-limiting influence. The trypsin inhibitor interferes with the liberation of methionine in the digestive tract and its effect is largely, if not entirely, negated by methionine feeding. The mechanism of the heat-stable inhibitor is unknown; its effect is counteracted partially and ir-

regularly by methionine but completely and consistently by the growth factor of cow manure.

Enzymatic treatment of raw soybean flour materially improved the nutritional value of this feedstuff. The hydrolysate did not quite equal the heated soybean oil meal, but in view of the entirely different origins of the 2 products, the results appear sufficiently similar to justify the statement that this experiment with enzyme-digested soybean flour confirmed the report by McGinnis and Menzies ('46) that papain digestion of raw soybean oil meal made it nutritionally equal to heated soybean oil meal. Hydrolysis, like heat-treatment, failed to overcome that portion of the growth inhibition which was counteracted by the growth factor of cow manure.

#### SUMMARY

Soybean oil meal fed to young chickens as 70% of the diet caused an inhibition of growth and increased mortality. Both of these effects were counteracted by the addition of the growth factor of cow manure to the diet but not by the addition of methionine.

The growth factor of cow manure improved the nutritional value of a chick diet containing raw soybean oil meal as the only protein concentrate, but not to the extent that it improved a diet containing heated soybean oil meal.

Evidence has been presented that soybean oil meal at high levels exerts an inhibiting effect on the growth of chicks which is not due to a heat-labile trypsin inhibitor, which is not nullified by heating or by enzymatic digestion, but which is counteracted by the chick growth factor of cow manure.

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# PROCEEDINGS OF THE ELEVENTH ANNUAL MEETING OF THE AMERICAN INSTITUTE OF NUTRITION

STEVENS AND CONGRESS HOTELS, CHICAGO,  
ILLINOIS, MAY 18-22, 1947

## COUNCIL MEETINGS

Council meetings were held at the Stevens Hotel on Sunday, May 18. All members were present. Formal actions of the Council are reported in the minutes of the business section.

## SCIENTIFIC SESSIONS

The scientific program consisted of 7 half-day sessions of scientific papers grouped according to topic. A total of 73 papers were presented and 9 were read by title. The abstracts of these papers were published in the Federation Proceedings, vol. 6, no. 1, March, 1947, pp. 401-424. If the number of papers increases much further it will be necessary to run simultaneous sections or to transfer papers to the list of those to be Read by Title.

## BUSINESS SESSIONS

Two business meetings were held, one at 8:30 Monday evening, May 19, following the Institute Dinner, and the other at 4:00 P.M. Wednesday, May 21, following the scientific program. These meetings were presided over by the President, A. H. Smith.

*Monday, May 19, 8:30 P.M.* The report of the Treasurer was presented by E. M. Nelson. The Auditing Committee (Dr. Elsa Orent-Keiles and Dr. Chester Tolle) reported that the

Treasurer's books were in order. The Treasurer's report was approved.

Dr. George R. Cowgill, Editor of *THE JOURNAL OF NUTRITION* gave a brief report concerning the Journal as follows:

Beginning with the January 1946 issue a return was made to the page format that was used before the war. This change was made with all of the journals published by The Wistar Institute. As usual, 2 volumes were published during the year, namely volumes 31 and 32; they contained 118 articles. During the year 153 papers were submitted for consideration. The average number of papers per issue was 10, compared with 8.5 for the previous year. The average number of pages per article this year, namely 12.4, was higher than the previous year's figure of 9.0 pages. Simple comparison of these figures is not really valid because the format had been changed to the older one with fewer words per page; on the other hand it should be remembered that the number of pages per volume has been increased from 100 pages to the newer limit of 120.

Volume 31, covering January to June 1946, inclusive, was designated the "John R. Murlin Honor Volume" in honor of Dr. Murlin who founded the Journal and served as its editor until 1939 when he became an emeritus member of the American Institute of Nutrition. It was arranged that the first issue, that for January 1946, should carry a picture of Dr. Murlin which could serve as a frontispiece for the volume. The issue also carried a short note of appreciation of Dr. Murlin written by his younger colleague Dr. E. S. Nasset. A discussion of the efforts made to honor Dr. Murlin in some way, which culminated in the plan to designate volume 31 as an Honor Volume, was given in last year's report and therefore need not be given here (*Fed. Proc.*, 5: 316, Sept. '46).

The printing shop difficulties that made it impossible during the war years for our printer, The Wistar Institute, to bring out each issue on or about the tenth of each month were gradually overcome during the year. The most recent issues show a vast improvement in this regard. The one for April, 1947, was only a few days late; that for May, 1947, appeared

on schedule. In last year's report a hopeful statement was made regarding the prospects in this regard. Inasmuch as the latest issue appeared on time, we have every reason to believe that this problem caused by the war has now been solved, which is of course very gratifying to all parties concerned.

The members approved the Editor's Report and extended to Dr. Cowgill and the Editorial Board a hearty vote of thanks for their service.

The appointment of Dr. Walter C. Russell as the representative of the Institute to the Division of Biology and Agriculture of the National Research Council and as Liason Officer to the Food and Nutrition Board was approved. The appointment of Dr. L. A. Maynard as our representative on the Committee on International Congresses was approved.

The President reported that the Executive Committee of the Federation had discussed the feasibility of reorganizing the administration of the Federation by the establishment of a permanent business secretary who would, among other duties, be responsible for the annual meetings, thus removing much of this burden from the local committees. The Placement Service (which Dr. H. B. Lewis can no longer supervise) might also be transferred to the office of the permanent secretary. Such a proposal would require an increase of Institute dues to \$4 (\$3 to the Federation; \$1 to the Institute). All of these recommendations were approved. The President also announced that the 1948 meeting would be held in Atlantic City.

It was voted that the Institute should have a dinner at the 1948 meeting.

Dr. Walter C. Russell, the Institute representative on the Committee on Organization of the American Institute of Biological Sciences, reported on the nature of the proposed new organization and indicated that the American Institute of Nutrition was eligible to become a charter member. After some discussion the invitation to join this new organization was declined on the basis that the Institute of Nutrition was



more closely related to the Federation Societies than to many of the proposed members of the American Institute of Biological Sciences.

The members observed a period of silence in honor of the following deceased members: Morris S. Fine, Charles L. Hoagland, Paul Roth, and Alfred Shohl.

*Wednesday, May 21.* The tellers reported that the following officers had been elected for 1947-1948:

President—R. M. Bethke  
 Vice-President—E. M. Nelson  
 Treasurer—N. R. Ellis  
 Councillor—A. D. Holmes  
 Associate Editors—E. W. Crampton  
                           O. L. Kline  
                           R. W. Swift

The following new members were elected on recommendation of the Council:

Aaron Arnold	Seth Roth Johnson
J. C. Bauernfeind	Francis G. McDonald
Herbert R. Bird	Mary Elizabeth Reid
Alex Black	H. E. Robinson
David K. Bosshardt	Saul Rubin
George M. Briggs	H. P. Sarett
W. W. Cravens	H. A. Schneider
Eva Donelson	Janice M. Smith
D. V. Frost	Clara A. Storvick
Ross A. Gortner	Madelyn Womack
B. Connor Johnson	

The President announced that a Joint Committee on Nomenclature had been established in cooperation with the American Society of Biological Chemists. The committee consists of:

C. A. Elvehjem, Chairman  
 E. M. Nelson  
 A. D. Welch  
 H. J. Almquist

This action was approved.

Dr. Griffith presented to the Institute the essential content of bills currently under consideration by Congress (HR 3215 and Senate Bill 504) regarding the status of professional men

(other than physicians, dentists, and veterinarians) in the Medical Departments of the Army and Navy. These bills would group such professional men in a heterogeneous group of professional and non-professional workers (the Medical Service Corps). The defects of such a set-up were pointed out by Dr. Griffith. The Instituté voted to send a protest to the House and the Senate regarding this situation and the President appointed a committee consisting of Drs. Griffith, Nasset, and Carter to frame appropriate letters. (These were sent to the chairmen of the House and Senate Committees on Armed Services on May 29.)

President Smith appointed the following Nominating Committee for 1947-1948:

Harold Goss, Chairman  
H. G. Day  
L. A. Maynard  
H. B. Pierce  
Pearl Swanson

The Institute gave a hearty vote of thanks to the Local Committee for their fine service in organizing and arranging the meeting.

The meeting adjourned at 4:45 P.M.

#### DINNER AND PRESENTATION OF AWARDS

For the first time since the war the Institute held a dinner meeting (Hotel Continental, May 19). Following the dinner Dr. Murlin gave a very interesting talk regarding the early history of the American Institute of Nutrition. At this time also the presentation of the Institute Awards was made. Dr. L. A. Maynard as recipient of the Borden Award received a medal and a check. Drs. Paul Day, W. J. Darby, and E. L. Stokstad as co-recipients of the Mead Johnson and Company Prize received scrolls and checks.

Respectfully submitted,

H. E. CARTER, *Secretary*  
American Institute of Nutrition



# PRODUCTION OF NIACIN DEFICIENCY IN RATS

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(Received for publication March 17, 1947)

Krehl and coworkers ('45a) have reported that rats fed a low-protein diet containing 40% corn developed a growth depression which was correctable by either niacin or tryptophane. These findings have been confirmed by Dann ('46), Singal et al. ('46), and Spector and Mitchell ('46).

Tryptophane and niacin are apparently interchangeably active in reversing growth retardations produced by certain diets in chicks (Briggs, '45), in rabbits (Wooley, '47), and in dogs (Hundley, '46).

Dann ('46) has shown that the corn grits diet of Krehl et al. ('45b) will sharply reduce the urinary output of N<sup>1</sup>-methyl-nicotinamide (NMN) in the rat. It has been reported that the addition of tryptophane to various diets will cause a large increase in the urinary excretion of nicotinic acid and its derivatives in rats (Rosen et al., '46; Singal et al., '46), in horses and cotton rats (Schweigert et al., '47), and in man (Sarett and Goldsmith, '47; Perlzweig et al., '47).

These reports seem to indicate that tryptophane is an important precursor of niacin synthesis and that a niacin deficiency is produced when the diet contains insufficient tryptophane to compensate for the lack of preformed niacin.

However, Woolley ('46b) has prepared a concentrate from corn which has a "pellagrigenic" action in mice. In addition he has reported ('46a) that either niacin or tryptophane will

reverse the biological effects of 3 acetylpyridine. Kodicek et al. ('46) have reported that indole-3-acetic acid will produce the same growth depression in rats as will corn itself.

Groschke and Briggs ('46) and Krehl et al. ('46b) have found that certain amino acids have a marked growth-inhibiting action which can be overcome by niacin.

The present paper presents a study of the production of niacin deficiency in rats using diets free of any known "anti" factor, in which the only apparent etiologic agent was a relative lack of tryptophane. This has been possible by feeding a low percentage of casein supplemented with cystine or other tryptophane-deficient protein material.

#### METHODS

Male rats weighing 35 to 40 gm were placed individually in wire mesh cages at weaning and distributed to form groups according to weight and litter.

The diets used were modeled after the basal diet of Krehl et al. ('45) and consisted of casein (vitamin free) 15, sucrose 78, corn oil 3, salts (Osborne and Mendel) 4, and 1(—)cystine 0.15 gm. Vitamins were incorporated in the ration in the following amounts: thiamine 0.2, riboflavin 0.3, pyridoxine 0.25, calcium pantothenate 2, choline 100, inositol 10, 2-methylnaphthoquinone 0.1, biotin 0.01 and pteroyl glutamic acid 0.1 mg %. The niacin content of these diets was practically zero and niacin was added only when indicated. The casein or other protein material was varied from 6 to 40% at the expense of the sucrose. 1(—)cystine was added to most of the diets so that the cystine-methionine deficiency of casein was at least partially corrected, making tryptophane the principal amino acid limiting growth (Mitchell and Block, '46).

Growth periods varied from 3 to 8 or more weeks. Niacin tissue assays were done using *L. arabinosus* with a slight modification of the Snell and Wright ('41) method. Urines were collected under toluene and the N<sup>1</sup>-methylnicotinamide estimated promptly using the method of Huff et al. ('45).

RESULTS

*I. Effect of niacin at various levels of casein  
(plus cystine)*

The growth of rats on diets containing 6 to 40% casein was compared at each level of casein with and without added niacin. 1(—)cystine 0.15% was added to all diets.

Representative data are shown in table 1. The addition of niacin caused growth stimulation at each level of casein up to 20%. Statistical analysis however showed that only the niacin growth stimulation in 12% casein diets was certainly significant, there being less than 1 chance in 100 that the growth stimulation was accidental.

TABLE 1

*Growth and niacin tissue levels as influenced by diet and supplemental niacin.*

DIET <sup>1</sup>		GROWTH			NIACIN CONTENT OF TISSUES <sup>2</sup>		
Casein	Niacin	No. rats	Av. gain	t value <sup>2</sup>	No. rats	Liver	Muscle
%	mg %		gm/wk.			µg/gm	µg/gm
6	0	5	1.1				
6	2	5	1.9	0.95			
9	0	9	10.1		5	128 (115-155)	56 (39-73)
9	2	9	13.7	1.75	5	133 (110-157)	82 (75-94)
12	0	10	15.4		5	140 (120-173)	49 (40-70)
12	2	10	21.3	2.91	5	175 (135-212)	84 (79-93)
15	0	5	23.9		4	186 (165-200)	59 (58-60)
15	2	5	30.9	2.02			
20	0	9	28.8		4	172 (165-177)	82 (80-85)
20	2	9	30.0	0.70	4	181 (170-188)	84 (81-87)
30	0	3	26.9				
30	2	3	28.2	0.90			
40	0	4	29.7	0.37			
40	2	4	29.1				

<sup>1</sup> All diets included 1(—)cystine 0.15%.

<sup>2</sup> Calculated according to Fisher's "Statistical Methods for Research Workers." The author is indebted to W. M. Gafafer for these analyses.

<sup>3</sup> The author is indebted to Dr. J. G. Wooley for these assays. Values within parentheses indicate range.

In other experiments rats have been kept on these diets for 4 weeks with growth results comparable to those shown in table 1. At the end of this period the diets were reversed at each casein level so that rats not receiving niacin now received it, and vice versa. In the 9 and 12, but not in the 20% casein groups, growth shifts in favor of the niacin groups appeared.

In another experiment the effect of added tryptophane was found to be similar to that of added niacin. Using the 12% casein ration and 4-week growth periods, 4 rats grew at the rate of 13.9 gm/wk. When 10 mg % niacin was added to the same diet using 4 litter mates, growth was at the rate of 16.8 gm/wk. Four hundred mg % dl-tryptophane in 4 additional litter mates increased the growth rate to 17.1 gm/wk., but when both niacin and tryptophane in the above amounts were added to a fourth group of litter mates no growth stimulation was evident. The reason for the latter observation was not clear.

## *II. Liver and muscle niacin levels*

A close correlation was apparent between the tissue niacin levels and the percentage of dietary protein (table 1). Using the rats receiving 20% casein as normal, the 9 and 12% casein rats showed a reduced concentration of tissue niacin. The addition of 2 mg % niacin to the diet brought all muscle levels to normal and brought the livers to normal in the 12% but not in the 9% groups. The same amount of niacin did not alter the levels significantly in the 20% casein group.

## *III. Urinary N<sup>1</sup>-methylnicotinamide excretion*

Normally rats appear to excrete a large part of their known urinary niacin derivatives in the methylated form (Huff and Perlzweig, '42; Rosen et al., '46), and since the amount of N<sup>1</sup>-methylnicotinamide excretion bears a rather constant ratio to the amount of other niacin compounds, the excretion of this substance should give a good index of the total excretion of niacin.

The data (table 2) show a close correlation with the growth and tissue niacin assay results. If the excretion of the rats receiving 20% casein is taken as normal, then the 9 and 12% casein rats showed a marked reduction in N<sup>1</sup>-methylnicotinamide. The addition of 2 mg % niacin caused a rise to approximately normal levels in the 9 and 12% rats but had no consistent effect at 20% or more casein. The addition of 100 or 400 mg % dl-tryptophane to the 9 and 12% rations caused a very large increase in the excretion of N<sup>1</sup>-methylnicotinamide.

TABLE 2  
N<sup>1</sup>-methylnicotinamide (NMN) excretion.

DIET <sup>1</sup>			NO. OF OBSERVATIONS	NMN EXCRETION PER 24 HOURS	
Casein	Added			$\mu\text{g}/100 \text{ gm}$ body weight	Range
	Niacin	dl-Trypto- phane			
%	mg	%			
9	0	0	4	29	(19-55)
9	2	0	4	204	(128-315)
9	0	100	4	429	(189-633)
12	0	0	27	29	(6-75)
12	2	0	15	127	(16-360)
12	10	0	11	174	(100-268)
12	0	100	4	444	(277-607)
12	0	400	16	1074	(300-1980)
12	10	400	8	945	(365-1880)
20	0	0	5	121	(27-371)
20	2	0	6	112	(20-172)
30	0	0	4	90	(27-120)
30	2	0	5	97	(31-126)
40	0	0	4	195	(26-504)
40	2	0	4	223	(117-410)
Stock <sup>2</sup>	0	0	4	431	(246-828)

<sup>1</sup> All diets contained added l(—)cystine 0.15%.

<sup>2</sup> Ground Purina Dog Checkers.

#### iv. Other tryptophane deficient diets

The diets mentioned thus far seem to produce only a mild deficiency as judged by growth. After 8 to 10 weeks on the 9



or 12% rations, the response to niacin is no longer consistent. Also there is considerable litter variation and controls must be used in each case. Therefore these diets are not entirely suitable for routine use in studying niacin deficiency.

Other diets were constructed in which the relative deficiency of tryptophane was greater. In each instance rats on these diets as shown in table 3 showed a marked depression of

TABLE 3

*Diets for production of moderate to severe niacin deficiency in rats.*

CASEIN	TRYPTOPHANE DEFICIENT PROTEIN	TOTAL PROTEIN GM %	NIACIN MG %	ADDED DL TRYPTO- PHANF MG %	TOTAL TRYPTO- PHANF IN DIET MG %	GM/WE. GMS	AMN <sup>1</sup> IAC. μg/DAY
9 gm %	+ 9 Ess. Am. Ac. <sup>2</sup> 7.9 gm %	16.9	0	0	108	2	7
9	+ "	16.9	10	0	108	18	—
9	+ 10 Ess. Am. Ac. 8.1 gm %	17.1	10	200	308	17.5	782
9	+ Acid hydrolyzed Casein 4 gm %	13	0	0	108	6	
9	+ "	13	10	0	108	20	
9	+ Oxidized casein <sup>3</sup> 5.4 gm %	14.4	0	0	108	3.5	
9	+ "	14.4	10	0	108	18	
9	+ Gelatin 3 gm % and 1(—)cystine 0.15%	12	0	0	108	3.5	7
9	+ "	12	10	0	108	17.5	354
9	+ "	12	0	75	183	18.2	447
9	+ " 5.4 gm %	14.4	0	0	108	4	
9	+ " "	14.4	10	0	108	23	
9	+ " 9 gm %	18	0	0	108	1.8	10
9	+ " "	18	10	0	108	15.4	306
10	+ Gelatin + Am. Ac. 10 <sup>4</sup>	20	0	0	120	2	
10	+ "	20	2	0	120	25	
10	+ "	20.2	0	200	320	31	
plus tryptophane							

<sup>1</sup> N<sup>1</sup>-methylnicotinamide.

<sup>2</sup> Essential amino acids added in same amounts as given by Rose ('38) for meeting minimum requirements of rats.

<sup>3</sup> Prepared according to Toennies ('42).

<sup>4</sup> Amino acids added so as to be equivalent of 20% casein with amino acids except tryptophane.

growth which was correctable by either tryptophane or niacin. The use of tryptophane and niacin together did not increase the growth response over that when either one was used alone in optimum amounts.

# DISCUSSION

From the data presented it seems clear that when growing rats are maintained on a ration in which tryptophane seems to be the chief amino acid limiting growth, a marked diminution in niacin synthesis occurs with a resultant niacin tissue deprivation and depressed growth. When tryptophane is added to the diet, either as casein or tryptophane itself, an increase in niacin synthesis occurs.

These facts strongly indicate that tryptophane is the dietary precursor of niacin. Similar conclusions have been published by Singal et al. ('46), Rosen et al. ('46) and Schweigert et al. ('47).

The action of niacin in promoting growth in the presence of an apparent deficiency of tryptophane has been explained by Krehl et al. ('46a) as being due to increased efficiency of utilization of tryptophane. A similar conclusion was reached by Spector and Mitchell ('46) as the result of a paired feeding study.

However, it is also possible, and seems more likely to the writer, that the niacin acts to "spare" tryptophane. Since tryptophane seems to be the dietary precursor of niacin, a certain portion of it must be used up in this process. If niacin is supplied preformed, the tryptophane may be spared of this function, making it available for tissue building. Some evidence in this direction is noted in table 3 since with increasing amounts of casein and a constant amount of niacin, there was no notable increase in excretion of N<sup>1</sup>-methylnicotinamide (NMN). On the other hand, without niacin in the diet the NMN excretion varied roughly with the per cent of casein at least up to the level where growth was independent of the niacin intake. This observation could be explained by assuming that with adequate preformed niacin in the diet, the trypto-

phane is metabolized along with the other amino acids in a manner not leading to niacin synthesis. However, when a large amount of tryptophane is added, more than can be used in tissue building because of the limited supply of other amino acids, niacin is formed in excess even in the presence of adequate dietary niacin.

Since the diets used in this study were probably free of any "toxic" factors (with the exception of the gelatin diets) it seems evident that the primary etiology in the production of a niacin deficiency in rats is not an "anti" or "toxic" substance. If such factors have any role at all it must be only a contributory one. It is to be noted that in the report by Kodicek et al. ('46) on the use of indole-3-acetic acid, the basal diet used was 10.5% casein plus 0.15% cystine, a diet which by itself would likely produce a mild niacin deficiency according to the data reported in this paper.

#### CONCLUSIONS

Growing rats, maintained on a basic low-casein diet in which tryptophane was the chief limiting amino acid, developed a condition characterized by growth depression, diminished tissue niacin levels, and diminished excretion of N<sup>1</sup>-methylnicotinamide.

This condition was interchangeably corrected by either tryptophane or niacin.

Deficient rats treated with tryptophane showed a marked increase in niacin synthesis.

The possible mechanisms involved are discussed.

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# A BIOASSAY FOR PROTEINS AND PROTEIN DIGESTS

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THREE FIGURES

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In the course of large scale preparation of enzymatic digests of lactalbumin,<sup>3</sup> it was necessary to investigate the nutritional quality of the product for evaluation of its probable usefulness in human nutrition and as a routine control of the manufacturing procedure.

A method of rat bio-assay was devised which requires only 2 weeks to obtain a result, requires a minimum of chemical analyses, satisfies the dictum that accuracy of bio-assays is increased by comparing test samples with a standard substance and simulates the conditions of the greatest usefulness of protein digests in human nutrition, i.e., tissue regeneration after a period of negative nitrogen balance.

Casein was chosen as the standard to which other proteins or protein hydrolysates were compared as it is readily obtainable, inexpensive, and amino acid analyses of different samples have shown remarkably good agreement (Dunn et al., '46). Vitamin test casein GBI<sup>4</sup> was the particular brand used.

The basis of the assay is the comparison of the amount of dietary nitrogen in the form of casein with the amount of

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<sup>3</sup> Lactamin, Wyeth, Incorporated, Philadelphia, Pa.

<sup>4</sup> General Biochemicals, Inc., Chagrin Falls, Ohio, 14.3% nitrogen.

other forms of dietary nitrogen per 24 hours required to maintain an adult rat for 1 week with no loss or gain in body weight, after a preliminary period of 1 week on a protein-free diet, caloric intake being held constant. The nutritional quality of the nitrogen source being tested is then numerically expressed by the ratio of the weight maintenance level of casein nitrogen over that of the nitrogen source being tested, casein nitrogen being assigned a nutritional index of unity.

## EXPERIMENTAL

### *Test procedure*

After completion of preliminary experiments the procedure adopted was that described in the following paragraphs.

Seventy or more male Wistar rats of approximately the same age, weight and previous history were selected for assay groups. The weight varied from 180 to 300 gm. In any one test the variation between the weights of the rats was within 50 gm.

The rats were individually caged in a constant temperature room maintained at  $23^{\circ} \pm 1^{\circ}\text{C}$ . Weighed amounts of food were fed in containers which could not be easily upset. Any spilled food was recovered, weighed, and an equivalent amount given to the rat at the next feeding. Any rat that persistently spilled his food was excluded. Water was supplied *ad libitum*. Separate supplements of 25  $\mu\text{g}$  thiamine hydrochloride, 40  $\mu\text{g}$  riboflavin, 25  $\mu\text{g}$  pyridoxine, 100  $\mu\text{g}$  calcium pantothenate and 20 mg of choline chloride were given daily and 1 mg of  $\alpha$ -tocopherol and 0.3 mg of vitamin K once weekly. The feeding and weighing of the rats were done at the same hour every day.

The assay interval consisted of 2 periods — the depletion period and the test period — each of 1 week's duration. The daily allowance of ration, hence caloric and nitrogen intake, was fed in amounts proportional to the average surface area of the test animals at the start of the assay. The amount of

the diet to be fed was calculated to the nearest gram on the basis of 2.35 gm (11 cal.) per square decimeter of surface area. Surface area =  $11.36 \times \text{weight}^{\frac{2}{3}}$  (Carman and Mitchell, '26).

The diet fed during the 7-day depletion period had the following composition: cerelese 79, hydrogenated cottonseed oil <sup>5</sup> 15, salt mixture  $\frac{1}{4}$  (U.S.P. no. 2) and cod liver oil U.S.P. On the eighth day, the rats received the usual amount of protein-free diet at 9 a.m. At 11.00 a.m. the rats were weighed and at the same time any uneaten food removed from the cage. This procedure was found to give the most consistent weights. Some of the rats had eaten their ration within the first few hours on the previous day and consequently would have been in a fasting state unless fed on the following morning before the time of weighing. Without this additional feeding the weights of the rats varied considerably, depending on how rapidly the diet on the seventh day had been consumed. The weight loss with this procedure in 12 groups of 70-100 rats varied from 8.6%  $\pm$  2.1% of the original weight. In any single experiment the average deviation was  $\pm$  0.7%.

Sixty-five of the depleted animals, selected on the basis of conformity to the average weight drop, food habits in regard to food spillage and consumption of all the diet, were divided by weight into 13 groups of 5 each. With these 13 groups, 3 test substances and a casein standard could be compared.

One group of 5 was continued on the protein-free diet. Three groups of 5 were fed diets containing 0.36, 0.72 and 1.08% casein nitrogen which is equivalent to 2.5, 5.0 and 7.5% casein. The remaining 9 groups received equivalent amounts of nitrogen obtained from each of the 3 protein materials being tested. These nitrogen levels were chosen because under the conditions of the experiment the weight change values obtained were found to fall in a suitable range for interpolation of the value of the amount of nitrogen fed daily which would cause neither loss or gain from the depleted weight.

<sup>5</sup> Crisco.



The dried test substances<sup>6</sup> were incorporated into the protein-free diet at the expense of the cerelose, so that all diets were very nearly iso-caloric, containing 47 cal. per gm of diet.

These diets were then fed on the eighth day at about 2 p.m., several hours being required to divide the rats into groups, and from then on for 6 more days. On the fifteenth day the animals were fed at 9:00 A.M. and weighed finally at 11:00 A.M. The rats were returned to the stock diet, supplemented with whole milk powder, for 2 or 3 weeks before being used for assay.

A plot was made of the change in weight of each group from the end of the depletion period to the end of the test period against the milligrams of nitrogen fed daily. The amount of nitrogen of each protein or digest necessary to maintain a constant weight in the depleted rats was taken from the graph; this was the point at which the curves crossed the point of zero weight change. Nutritional indexes were then calculated.

### *Preliminary experiments*

The weight changes of protein-depleted rats fed various levels of casein were investigated. Seven groups of 5 rats each (average weight, 220 gm) were depleted of protein for 1 week. One group was continued on the protein-free diet, the other 6 groups received 10 gm of diets containing respectively, 0.29, 0.57, 0.86, 1.15, 1.43 and 1.72% casein nitrogen. The groups were weighed daily for the next 12 days and the average daily weight change from the weight at the end of the depletion period was plotted against the daily nitrogen intake. The curves for several of the days are presented in figure 1. It may be seen that an approximately straight-line relationship was obtained in the regions of low nitrogen intake. The amount of daily nitrogen sufficient to maintain a constant weight was interpolated from each curve. Values obtained on the fifth to the twelfth days (excluding the tenth

<sup>6</sup> Hydrolysate solutions presented for assay were dried from the frozen state.

day) were 82, 85, 80, 78, 79, 80 and 81 mg of casein nitrogen (average 80.8) required per day for weight maintenance. Since a constant value was obtained from at least the fifth day on, it was decided that a 7-day test period would be of sufficient duration.

The validity of body weight changes, as a measure of protein nitrogen utilization under the conditions described in the above section, was investigated by a series of nitrogen balance studies patterned after those used by Melnick and

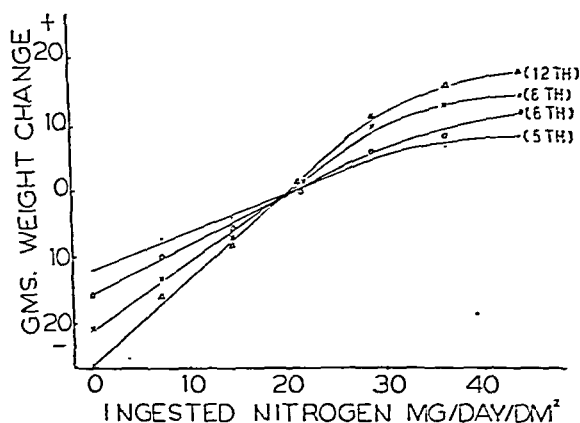


Fig. 1 The weight change of protein-depleted rats fed varying amounts of casein nitrogen. (The number in the parenthesis indicates the number of days of feeding.)

Cowgill ('37) in the determination of the minimal protein requirement for the establishment of nitrogen equilibrium in dogs. Rats from groups being fed casein diets were placed in metabolism cages and urine and feces were collected for the last 4 days of the assay test period. The average nitrogen balances for the 4-day periods and average changes in weight during the test period were plotted against daily nitrogen intake. The results of the typical experiment, presented in figure 2, show close positive correlation between nitrogen balance and body weight change with respect to daily nitrogen ingestion, hence it may be assumed that the weight changes observed are valid criteria of comparative nitrogen utilization.

tion. In all experiments, the daily amount of nitrogen required for nitrogen balance equilibrium was less than necessary for weight maintenance.

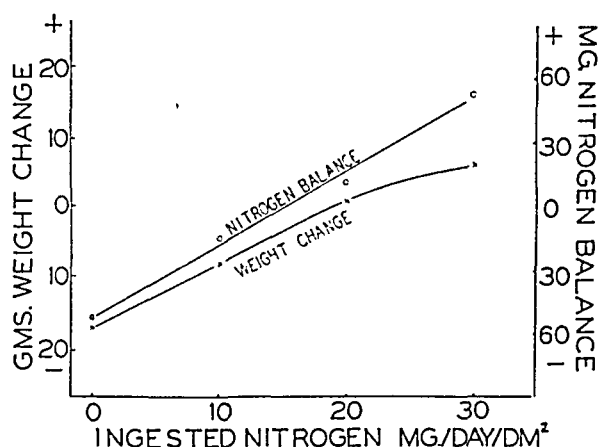


Fig. 2 The relationship of nitrogen balance and body weight change of protein depleted rats to ingested casein nitrogen. (Each point is an average of 5 values.)

TABLE 1

*Assays of individual proteins and digests.*

PROTEIN OR DIGEST	NUTRITIONAL INDEX
Casein (SMACO vitamin test)	1.00
Lactalbumin (Labeo)	$1.61 \pm 0.08^1$ (6) <sup>2</sup>
Pancreatic digest of lactalbumin	$1.44 \pm 0.07$ (13)
Crystalline Edestin	0.78, 0.73
Casein + 2% dl-methionine	1.65
Pepsin digest of liver protein	1.18
Equal parts of casein and lactalbumin	1.24

<sup>1</sup> Average deviation.

<sup>2</sup> Number of determinations in parentheses; otherwise 1 determination.

## RESULTS

The assay procedure described was used routinely to evaluate the nutritional quality of commercial batches of pancreatic digests of lactalbumin. The digests were found to be of uniform nutritional quality (table 1). In table 1 is also pre-

sented a list of nutritional indexes found for several other proteins and protein digests by this method of assay.

The results of a typical assay are presented in figure 3. In this assay a sample of lactalbumin digest, undigested lactalbumin, and a sulfuric acid hydrolysate of casein were compared with the casein standard. The daily amounts of nitrogen required for weight maintenance in mg/day/dm<sup>2</sup> were casein 20.9, lactalbumin 12.6 and lactalbumin digest 15.8, respectively. The nutritional indices were calculated to be 1.58 for lactalbu-

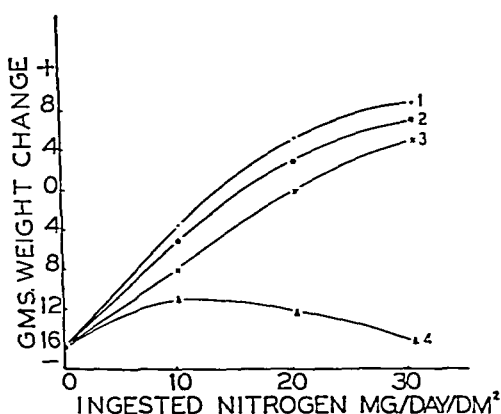


Fig. 3 Body weight change of protein-depleted rats when fed varying levels of casein, lactalbumin, pancreatic digest of lactalbumin and acid hydrolysate of casein. o—casein (curve 2); pancreatic digest of lactalbumin (curve 1); x—lactalbumin (curve 3); acid hydrolysate of casein (curve 4).

min and 1.39 for lactalbumin digest. In this and other tests the enzymatic digests of lactalbumin, either commercial or laboratory preparations, while always nutritionally superior to casein, were found to be inferior to undigested lactalbumin.

The weight change curves are typical of the results constantly obtained (higher nitrogen intake causes increase of weight) except in the case of the acid hydrolysate of casein. This product, fed in a small amount (approximately 10 mg N/dm<sup>2</sup>/day), does not cause a weight loss as large as does a diet entirely devoid of protein nitrogen; however increasing amounts of nitrogen lead to a progressively greater weight

loss. From these results it would seem possible that a large amount of protein hydrolysate nitrogen deficient in 1 or more amino acids would be more harmful to the animal than a diet entirely free of nitrogen. Such an effect has been reported by Frazier et al. ('47) who employed experimental diets composed of mixtures of crystalline amino acids.

In the course of this investigation 4 large groups of rats were maintained for assay purposes. Each group was used for 2 or 3 assays before being discarded. One group was subjected to 6 successive assays to determine if the increasing age and weight of the animals and repeated stresses of the procedure would affect the value obtained for the amount of casein nitrogen required for weight maintenance.

In table 2 are presented the experimental values obtained for casein and lactalbumin using the various assay groups. The weight maintenance level of nitrogen was not found to be

TABLE 2  
*Nitrogen requirement for weight maintenance of rats partially depleted of protein.*

ASSAY GROUP	SUCCESSIVE ASSAYS	AVERAGE BODY WEIGHT AT DEPLETION	DAILY RATION ALLOWANCE	MG N/DAY/DM. <sup>2</sup>	
				Casein	Lactalbumin
		gm	gm		
I	1	189	10	21.2	...
	2	205	10	21.0	...
	3	221	11	20.9	12.6
II	1	175	10	25.0	...
	2	197	10	22.6	...
	3	210	10	24.1	13.3
	4	228	11	22.8	...
	5	236	12	20.1	...
	6	254	13	21.6	13.3
III	1	208	10	20.0	13.1
	2	224	11	20.1	...
IV	1	168	10	20.6	12.0
	2	228	11	20.2	12.9
Average				21.6 ± 1.1	12.9 ± 0.4

dependent on the group of rats used or to be affected by the number of times up to 6 that the group has been used. The average value of all the determinations was  $21.6 \pm 1.2$  mg of casein nitrogen/day/dm<sup>2</sup>. The average value for lactalbumin was  $12.9 \pm 0.4$ . The average deviation of approximately  $\pm 5\%$  indicates that under these experimental conditions consistent results may be obtained.

### DISCUSSION

By subjection of the assay animals to a state of protein depletion, a condition is created which simulates that present in human patients requiring dietary protein therapy. Depletion of the rats' body stores of protein and the feeding of dietary protein at sub-optimal levels probably enhances the sensitivity of the assay, since the nutritional economy of the animals would require that the protein nitrogen be maximally utilized for the more essential processes of tissue maintenance. Under these conditions the responses produced by proteins or digests of different nutritional characteristics are emphasized. The response produced by each test sample is compared with that produced by a casein standard, thus neutralizing extraneous factors and allowing less exact specifications of the weight, age and breed of the rats employed. Since the response is evaluated with respect to the amount of nitrogen fed, the nutritional index is a measure of the digestion, absorption and utilization of the protein test substance.

Each group of rats may be used for several assays, provided a repletion period of 2 or 3 weeks is permitted between assays. The increasing size of the animal in subsequent assays is compensated for by increasing the daily dietary caloric and protein nitrogen allowance and by calculating the nitrogen maintenance requirement on the basis of surface area. The simplicity of the method recommends it for routine assay purposes. The 2-week assay period is relatively short for an animal assay. The only manipulations are those of weighing the rats and the daily rations; chemical analysis is limited

to that of the determination of nitrogen in casein and in the proteins or protein digests being tested.

#### SUMMARY

An assay for the evaluation of the nutritional quality of proteins and protein digests has been described. The assay is based on the determination under standardized conditions of the amount of fed protein or protein digest nitrogen necessary daily to maintain a constant weight in adult rats that have been partially depleted of body protein. Rats are fed a protein-free diet for 1 week; various groups are then fed varying levels of each test sample for the second week. The daily allowance of ration is proportional to the average surface area of the rats. By plotting body weight change during the second week against daily nitrogen intake, it is possible to interpolate the amount of nitrogen necessary to maintain constant weight in the partially depleted animal. Each test substance is evaluated by comparison with a casein standard.

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# THE NUTRITION OF THE MOUSE

## I. A DIFFERENCE IN THE RIBOFLAVIN REQUIREMENTS OF TWO HIGHLY INBRED STRAINS<sup>1</sup>

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ONE FIGURE

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The riboflavin requirements as well as the symptoms of riboflavin deficiency have been described for many species of laboratory animals. Bessey and Wolbach ('39) reported in some detail the cutaneous and ocular lesions shown by rats deficient in this vitamin. In the same year Street and Cowgill ('39) published results obtained with dogs maintained on a synthetic, riboflavin-deficient diet. These workers found that a daily supplement of 25  $\mu$ g of riboflavin per kg of body weight would maintain dogs in apparent good health for a considerable period of time. Lippincott and Morris ('41-'42) and Morris and Robertson ('42-'43) studied many aspects of the problem using mice of the C<sub>3</sub>H strain (highly susceptible to spontaneous mammary tumors). From a clinical viewpoint the monkey is a most interesting experimental animal. For this reason especially the work of Cooperman et al. ('45) deserves attention. These workers have concluded that the

<sup>1</sup> This work was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council. Some of the early experiments were carried out with funds from the Nutrition Foundation, Inc., and the Anna Fuller Fund.



monkey requires 25 to 30  $\mu$ g of riboflavin per kg of body weight, figures in good agreement with those of Street and Cowgill ('39) for the dog. Cooperman et al. ('45) have described the cutaneous and ocular changes seen in the riboflavin-deficient monkey. They also reported lowered red and white cell counts and hemoglobin levels. Kornberg, Daft and Sebrell ('45) described similar blood changes in riboflavin-deficient rats. It is rather interesting to note that the blood picture of the monkey could be restored to normal more readily when riboflavin was administered together with whole liver, and that in the rat the red cell count could be restored with riboflavin but that the granulocytopenia responded best to folic acid. Finally, Bird and associates ('46) have determined the riboflavin requirement of chicks and poults to be, respectively, 2.75-3.25 and 3.25-3.75  $\mu$ g per gm of food.

The mouse has long been known as an ideal laboratory animal for studies in genetics, cancer, infectious diseases, serology, etc. Yet by comparison with the rat very little is known of the specific nutritional requirements of the mouse. The need for quantitative information concerning the demand for riboflavin is enhanced by the possible relation of this vitamin (along with others, of course) to the cancer problem. The protective action of riboflavin against liver tumors induced by azo dyes has been demonstrated by Miller and associates ('41) and by Sugiura and Kensler ('41). While most of the azo dye work has been done with rats, due to the peculiar susceptibility of this species, one must consider the possibility that riboflavin may exert a similar protective action in the mouse, which is susceptible to o-amino-azotoluene as shown by Andervont and Edwards ('42-'43) and by Kirby ('45). Morris and Robertson ('42-'43) have studied the retarding effect of riboflavin-deficient diets on the growth of spontaneous mammary tumors.

In view of the need for precise information about the nutritional requirements of mice it was decided to study their rates of growth on several synthetic diets differing only in their content of riboflavin. Since Rogers, McElroy and Cowgill

('42) had noted the existence of strain differences in the nutritional requirements of mice, this investigation was carried out with 2 highly inbred strains — C<sub>57</sub> (low incidence of spontaneous mammary tumors) and A (high tumor incidence).

#### EXPERIMENTAL

Five synthetic diets differing only in their riboflavin content were used in this study. Diet 120 had the following percentage composition: "vitamin-free" casein 30, hydrogenated cottonseed oil<sup>2</sup> 15, roughage<sup>3</sup> 3, salts<sup>4</sup> 7, dextrose C.P. 45, linoleic acid 0.41, and cod liver oil concentrate<sup>5</sup> 0.30. Each 100 gm of ration also contained the following vitamins:  $\alpha$ -tocopherol 9 mg, choline 150 mg, inositol 100 mg, p-aminobenzoic acid 100 mg, calcium pantothenate 3 mg, nicotinic acid 1 mg, thiamine 0.5 mg, and pyridoxine 0.5 mg. While no riboflavin was added to this basal ration, it was found by fluorometric assay to contain as trace contamination 0.03 mg of this vitamin per 100 gm of diet. The other 4 rations used here differed from the basal in containing the following added riboflavin per 100 gm: no. 121 diet 0.2 mg, no. 122 diet 0.4 mg, no. 123 ration 0.6 mg, and the no. 124 diet 1.0 mg.

A total of 108 male mice of the C<sub>57</sub> and A strains, weaned when 21 days old, were used in these studies. The animals were placed in individual screen-bottom cages and fed diet 120 ("riboflavin-free") for 1 week. At the end of this depletion period they were divided into 5 groups which were fed, respectively, diets 120, 121, 122, 123, and 124. Care was taken to distribute litter mates as uniformly as possible. The cages were kept in an air-conditioned room maintained at 75°F. and 50% relative humidity. The animals were weighed daily (with a few exceptions in the early phases of the experiment).

To determine the effect of riboflavin-low diets on adult mice, males of the C<sub>57</sub> strain (16 to 18 weeks old, raised from

<sup>2</sup> Crisco.

<sup>3</sup> Ruffex.

<sup>4</sup> Sure's Salts no. 2.

<sup>5</sup> White's Cod Liver Oil Concentrate Liquid.

weaning on a synthetic diet high in riboflavin and similar in composition to no. 124) were placed on diets 120 and 121. These animals were weighed once a week.

In a number of instances food consumption was measured by placing 4.0 gm of the diet in a half-ounce glass jar into the cover of which a hole of  $\frac{3}{4}$ -inch diameter had been cut. A circular piece of  $\frac{1}{4}$ -inch mesh wire screen was laid loosely on top of the food. This piece was small enough to sink freely toward the bottom of the jar as the food was consumed. The entire jar assembly was placed in a 150-ml beaker. Usually in the course of 24 hours some food was spilled into the beaker and was weighed together with the residue in the cup. In another series of experiments it was found that food spillage outside the beaker was ordinarily so small as to be completely insignificant.

Red and white cell counts were done on blood obtained by clipping the tip of the tail; hemoglobin was determined by the alkaline hematin method with the aid of the Evelyn photoelectric colorimeter (Evelyn, '36). Riboflavin determinations on muscle and liver of 70 animals were carried out by the method of Peterson, Brady and Shaw ('43).

## RESULTS

A distinct strain difference became apparent in the growth response to the riboflavin-free diet during the 1-week depletion period. Mice of the  $C_{57}$  strain gained weight steadily during this interval while A strain mice gained less weight and even showed some weight loss before the end of the depletion period. In the first group of experiments (fig. 1 A) maximal growth was obtained with the  $C_{57}$  strain when the diet contained 0.4 mg of riboflavin per 100 gm of diet (shown graphically as open circles). On the other hand the A strain showed maximal growth only at a riboflavin level of 0.6 mg per 100 gm of diet (open triangles). In figure 1 A the results with diets 123 and 124 (0.6 and 1.0 mg level) were combined since they were identical.

To test further the apparent strain difference in riboflavin requirement more mice of both strains were placed for 1 week on the riboflavin-free diet (no. 120) after which they were fed diets 122 or 123 (0.4 and 0.6 mg of riboflavin per 100 gm of diet, respectively). The results are shown graphically in figure 1 B. The response of the 2 strains to the depletion

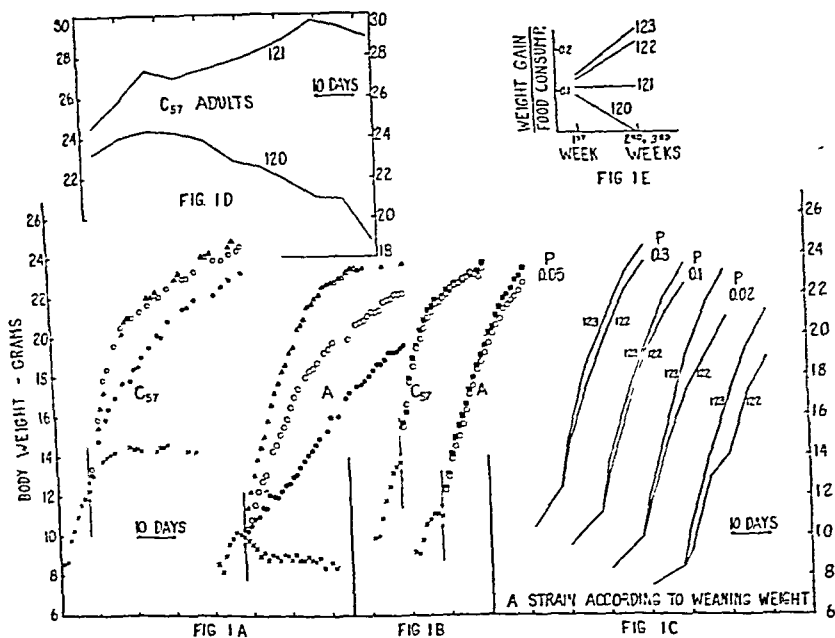


Fig. 1 Weights during the 7-day depletion period (the ends of which are indicated by arrows) are averages of all comparable groups regardless of subsequent riboflavin levels. The graphic symbols are as follows: diet 120  $\times \times \times$ ; diet 121  $\bullet \bullet \bullet$ ; diet 122  $\circ \circ \circ$ ; diets 123 and 124 combined  $\triangle \triangle \triangle$ ; diet 123  $\blacksquare \blacksquare \blacksquare$ . P values were calculated on the basis of total weight gains above the weaning weights. Mice shown in figure 1 A were followed until 70 days old, those in figure 1 B until 50 days old.

diet was identical with that observed in the first experiments (fig. 1 A). Mice of the C<sub>57</sub> strain again grew equally well on diets 122 and 123 while A strain animals grew better on the diet with the higher riboflavin level. In these experiments, however, the difference was small though significant as seen by the P value calculated for the respective increase in weight

from age 21 days to 50 days. To determine the reason for the large difference seen in figure 1 A and the small difference in figure 1 B shown by strain A on diets 122 and 123 these animals were grouped according to weaning weight (fig. 1 C). The animals fell into 4 groups: 7-7.9, 8-8.9, 9-9.9, 10 to 10.9 gm. In all 4 groups the animals receiving the higher riboflavin level grew better, but the difference is not statistically significant for the 2 groups of greatest weaning weight.

The efficiency with which the A strain mice utilized the various diets for body weight gain is shown in figure 1 E. The first point of each line represents the ratio of weight gain to food consumption during the initial week on the riboflavin-free depletion diet (no. 120). The final point on the line is the ratio computed for total weight gain and food consumption in the second and third weeks of the experiments during which the animals received diets of varying riboflavin content. The mice which were continued on the riboflavin-free diet showed during the second and third weeks of the experiments less weight gain per gm of food consumed (the ratio is actually slightly negative since the animals lost weight). On diets 122 and 123 the weight gain per gm of food consumed was greater than during the depletion period. Mice of the C<sub>57</sub> strain showed a considerably greater weight gain per gm of food consumed during the depletion week than did animals of the A strain.

When adult male C<sub>57</sub> mice were fed the riboflavin-free diet (no. 120), they continued to gain weight for a short time. Soon, however, they slowly declined in weight and gradually showed the lesions characteristic of riboflavin deficiency. During the eleventh week on this diet the weight loss became much more pronounced and during the tenth week the first death in this group occurred. Adult mice fed diet 121 continued to gain weight slowly. These results are plotted in figure 1 D.

The riboflavin content of muscle and liver is shown in table 1 together with red and white blood cell counts and hemoglobin levels. It is interesting to note that the A strain ani-

mals showed practically identical tissue riboflavin levels and blood pictures on diets 121, 122, and 123. On the other hand the C<sub>57</sub> strain reared on diet 121 had distinctly lower tissue riboflavin levels than when raised on diet 123. The C<sub>57</sub> mice on diet 121 also had lower red cell counts than did comparable animals on diets 122 and 123. The adult C<sub>57</sub> mice maintained on diets 120 and 121 had the lowest red cell counts, hemoglobin levels and muscle and liver riboflavin concentrations of all the groups studied.

TABLE 1

*Compilation of data on blood counts and the riboflavin content of muscle and liver.*

STRAIN	DIET	CELL COUNTS		HEMO- GLOBIN %	RIBOFLAVIN μG/GM WET TISSUE	
		RBC × 10 <sup>6</sup>	WBC × 10 <sup>3</sup>		muscle	liver
C <sub>57</sub> : young mice	121	9.35(5) <sup>1</sup>	10.2(4)	17.4(4)	3.5(5)	25(4)
	122	9.97(13)	9.3(12)	16.9(13)	4.4(14)	30(14)
	123	10.02(11)	7.3(11)	17.1(11)	5.3(11)	32(11)
A: young mice	121	9.23(6)	6.7(6)	15.1(6)	6.0(7)	33(6)
	122	8.98(19)	6.6(18)	15.1(18)	6.0(12)	33(14)
	123	9.02(17)	7.8(16)	15.1(17)	5.0(8)	33(7)
C <sub>57</sub> : adult mice	120	7.01(12)	6.8(11)	13.1(4)	2.7(2)	13(2)
	121	8.48(7)	8.5(7)		3.4(3)	17(4)

<sup>1</sup> The numerals in parentheses indicate the number of determinations.

## DISCUSSION

The relationship between weaning weight and the subsequent requirement for riboflavin emphasizes the importance of careful nutritional standardization of the breeding colony. Minimal variations in the weaning weights of mice may be expected when the breeding stock is maintained on a near-optimal diet. In our efforts to accomplish this we have, however, been struck by the cost of such rations when compared with commercial feeds which all too frequently cannot be called optimal. Our findings with respect to the importance of weaning weights are supported in part by the report of Sherman and Ellis ('39) that the progress of young rats on

riboflavin-deficient diets depended upon the maternal riboflavin intake even though this intake was 3, 7 or 10 times the minimal requirement. Clandinin ('46) found that the early growth rate of chicks was influenced by the maternal riboflavin intake. Although all animals in our breeding colony received the same ration, it may well be that the individual differences in lactation efficiency, which gave rise to different weaning weights of the young, were also reflected in different concentrations of riboflavin in the tissues of the young. Varying tissue vitamin levels at the start of the experiment may be expected to give rise to different growth rates especially on diets containing marginal or submarginal levels of riboflavin.

The reduced food intake of mice on riboflavin-deficient diets has been pointed out by Morris and Robertson ('42-'43) and others. In agreement with our results (fig. 1 E) Mannering and Elvehjem ('44) have reported poor utilization of such deficient diets for growth and maintenance. It is interesting to note that  $C_{57}$  strain mice on diet 122 (which just gives maximal growth on this type of ration) ingested in the neighborhood of 500  $\mu$ g of riboflavin per kg of body weight per day — which is roughly 20 times the level reported for the dog and the monkey. Such a difference in the per kg requirement of riboflavin for 2 species differing widely in body size agrees well with the calculations of Cowgill ('32) for the thiamine requirement.

The loss of body weight of adult mice kept on riboflavin-deficient diets for 9 weeks was found by Morris and Robertson ('42-'43) to be about one-third of the initial weight of  $C_3H$  animals. The loss shown by our  $C_{57}$  adults was found to be somewhat less. We have observed, however, in unpublished experiments that A strain mice under such conditions lost weight more rapidly than  $C_{57}$  animals. It may or may not be significant to mention that both the A and  $C_3H$  strains show high incidence of spontaneous mammary tumors whereas the  $C_{57}$  strain does not. The lesions we have observed have been adequately reported by Langston, Day and Cosgrove ('33), Lippincott and Morris ('41-'42), and by Jones

et al. ('45), and therefore the description need not be repeated here.

We have observed that A strain mice generally show lower cell counts than do C<sub>57</sub> mice. One may therefore consider all of the cell counts of the A strain on diets 121, 122 and 123 to be normal. On the other hand the red cell counts of young C<sub>57</sub> mice were definitely lower on diet 121 than at higher riboflavin levels. The close parallelism between liver riboflavin concentrations and red cell counts does not necessarily suggest a causal relationship. The amount of the vitamin per gm of liver reflects probably the quantity of riboflavin available for functions such as red cell formation. It is a matter of speculation whether the lower tissue riboflavin content of C<sub>57</sub> mice on diet 121 was due to the fact that these animals showed fairly rapid growth on this diet while the A strain showed only poor growth. This slower growth rate of the A strain may possibly leave more riboflavin available for red cell formation and permit greater tissue saturation at the lower levels of dietary riboflavin.

The strain differences which we have observed here with respect to the riboflavin requirement for growth, red cell formation, and tissue saturation do not necessarily have a direct bearing on the cancer problem. It would be premature to say that the higher vitamin requirements of the A strain determine its cancer susceptibility. It is, however, clearly evident that these observed differences may reflect diverse metabolic patterns, using the term in the widest possible sense, the sum total of which causes one strain to be cancer resistant and the other cancer susceptible.

#### SUMMARY AND CONCLUSIONS

1. The riboflavin requirement of 2 highly inbred strains of mice has been studied by measuring growth rates at various dietary levels of the vitamin after a 1-week depletion period.
2. Mice of the C<sub>57</sub> strain showed maximal growth under the conditions of these experiments when the diet contained



0.4 mg of riboflavin per 100 gm, while the A strain required a dietary level of 0.6 mg.

3. At an 0.2 mg level  $C_{57}$  mice had lowered red cell counts and muscle and liver riboflavin content, while the A strain showed no such difference.

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# COMPARATIVE NUTRITIVE VALUE OF CASEIN AND LACTALBUMIN FOR MAN

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ONE FIGURE

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The addition of methionine to a casein hydrolysate, or to a diet of mixed proteins does not increase the efficiency of nitrogen utilization in man (Cox et al., '47; Johnson et al., '47). Since such an addition to intact casein does improve nitrogen utilization in other species (Osborne and Mendel, '15), and since the processing to which a hydrolysate is subjected during the manufacturing process may cause it to differ from the original protein, it is essential to determine the nutritive value of whole casein for man, and to compare it with that of lactalbumin.

## EXPERIMENTAL OBSERVATIONS

The casein and lactalbumin employed in the study were commercial products.<sup>1</sup> Analyses for various constituents are summarized in table 1. The casein showed the expected deficiency in cystine but its methionine content and the cystine and methionine content of the lactalbumin, when corrected to a 16% nitrogen basis, agreed closely with the values given by Block and Bolling ('45). Each protein was fed to 20 rats at

<sup>1</sup> Sheffield Farms.

an intake level of 1.2% nitrogen and the difference in growth rate (table 1) clearly indicated that the samples were representative of casein and lactalbumin.

The study was made on 4 adult male volunteers: 3 laboratory workers and 1 clerical worker. All were found normal on the usual clinical examination, and continued their usual duties during the study. One subject contracted an epidemic

TABLE 1  
*Analytic comparison of casein and lactalbumin.*

	CASEIN	LACTALBUMIN
Total nitrogen, % <sup>1</sup>	13.46	12.31
Ash, % <sup>1</sup>	1.77	9.05
Fat, % <sup>1</sup>	1.47	.58
Calcium, % <sup>1</sup>	0.18	0.18
Phosphorus, % <sup>1</sup>	0.80	0.18
Lactose, % <sup>2</sup>	0.4	0.6
Cystine, % <sup>3</sup>	0.2	2.2
Methionine, % <sup>3</sup>	2.8	2.2
Growth of rats, gm/day <sup>4</sup>	1.66	2.74
Gain in wt. per gm N ingested	16.7	21.8

<sup>1</sup> We are indebted to Mr. L. N. BeMiller for these analyses.

<sup>2</sup> We are indebted to Dr. D. L. Morris for this determination.

<sup>3</sup> Analyses by Food Research Laboratories, New York. Cystine was determined by a colorimetric procedure: Block and Bolling ('45); see p. 152. Methionine was determined by a microbiological procedure: Stokes et al. ('45).

<sup>4</sup> Average for 20 rats on each protein fed at level of 1.2% nitrogen for 8 weeks. Diet: Lard, 9; salt mixture, 4; cod liver oil, 2; wheat germ oil, 1; vitamin mixture, 0.2; casein or lactalbumin, 8.92, 9.75; dextrin to make 100.

infection ("influenza") during the last period on lactalbumin, and it was necessary to terminate his part in the study. The subjects were depleted of their protein stores for a period of 12 days, since in an earlier study (Cox et al., '47) a 21-day protein depletion had demonstrated that a steady state of minimal nitrogen excretion was reached in 8 days, and that no good purpose was to be achieved by longer depletion. The protein depletion diet of Mueller, Fickas and Cox ('43) was

employed; the nitrogen intake from the low-nitrogen vegetables and fruits was limited to 0.008 gm nitrogen per kg; and the caloric intake was maintained at 40 cal. per kg body weight. The nitrogen content of coffee and the soft drink used was not considered in the intake and was subtracted from the urinary nitrogen.

A level of supplementation insufficient to produce nitrogen retention was intentionally chosen. It was thought that a continued small loss of nitrogen was preferable to retention for comparative purposes, since we wished to insure a maximal utilization of the ingested protein and maintenance of a reasonably steady condition of nitrogen storage. A further reason was that if positive balances should not be attained, the depletion periods could be used to calculate an average endogenous excretion. The supplementation level was 2% of the calories as the added protein, or 0.033 gm nitrogen per kg. This was 80% of the total protein intake, since 0.008 gm nitrogen per kg came from the basal diet.

In order to rule out the effect of antecedent conditions, each protein was fed alternately for two 4-day periods and each supplemented period was followed by a 4-day depletion period. Thus, there were 4 depletion periods of 4 days each from which the average endogenous urinary and fecal values could be calculated. It was feasible to use all of the depletion periods for this purpose since no storage of nitrogen occurred during supplementation.

Daily determinations of caloric and nitrogen intake (based on analyses of the actual foods employed), and urinary and fecal nitrogen were made. The standard macro-Kjeldahl method was employed for nitrogen, and the fecal samples were prepared for analysis by stirring with concentrated sulfuric acid.

The essential intake and excretion data are given in detail in table 2. The daily balance determinations have been averaged for the 4-day periods. It is readily apparent from the balance column that in none of the periods do the 4 daily balance values differ from the average value more than might

TABLE 2

*Urinary and fecal nitrogen excretions and balance during 12 days protein depletion and supplementation with casein and lactalbumin. Values are in grams.*

FOOD N INTAKE, GM SUPPLEMENTAL N, GM		SUBJECT A 0.740 3.053		SUBJECT B 0.574 2.366		SUBJECT C 0.711 2.934		SUBJECT D 0.522 2.155		N BALANCE	
Day		Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces	Day	Period
1		11.84	2.58	6.78	1.13	7.82	0.21	6.88	1.15	—8.94	
2		5.82	0.88	4.82	1.13	1.96	1.35	3.88	1.23	—4.60	
3		5.62	0.78	3.71	0.28	3.94	1.49	3.12	1.49	—4.47	
4		4.56	2.52	3.65	0.28	3.64	1.36	3.02	0.72	—4.30	
Period (1)	Av.	6.96	1.69	4.74	0.71	4.34	1.10	4.23	1.15		—5.58
5		4.31	0.66	3.53	0.28	3.65	1.78	2.88	1.16	—3.92	
6		4.40	0.66	2.86	2.50	3.70	1.78	2.82	1.36	—4.38	
7		3.41	1.19	2.98	1.47	3.07	1.02	2.67	1.13	—3.60	
8		3.09	1.70	2.14	0.57	2.59	1.12	2.81	0.91	—3.09	
Period (2)	Av.	3.80	1.05	2.88	1.21	3.25	1.43	2.80	1.14		—3.75
9		3.07	2.12	2.10	0.57	2.57	1.46	1.92	0.80	—3.02	
10		3.32	0.96	2.20	0.57	3.11	1.44	2.57	1.02	—3.09	
11		3.24	0.77	2.35	0.57	2.94	2.40	2.54	1.38	—3.46	
12		3.86	1.47	2.54	0.31	3.35	0.70	2.82	0.63	—3.32	
Period (3)	Ave.	3.37	1.33	2.30	0.51	2.99	1.52	2.46	0.96		—3.22
13	Casein	3.32	1.28	2.67	1.09	3.40	1.83	2.13	0.86	—0.91	
14		3.83	0.62	3.22	1.09	3.35	1.33	2.85	0.70	—0.98	
15		3.42	0.62	2.62	0.37	3.33	0.68	2.98	0.74	—0.43	
16		3.58	1.55	2.88	0.37	3.37	0.63	3.06	0.98	—0.84	
Period (4)	Av.	3.54	1.02	2.85	0.73	3.36	1.12	2.76	0.82		—0.79
17		4.05	2.21	3.89	0.37	4.05	1.27	2.84	0.92	—1.26	
18		3.33	1.12	2.75	0.31	2.54	2.24	2.67	0.77	—3.30	
19		3.19	1.17	2.96	0.31	2.77	1.20	2.95	1.00	—3.25	
20		3.25	1.05	2.87	0.36	2.53	0.30	2.19	0.73	—2.74	
Period (5)	Av.	3.46	1.39	3.12	0.34	2.97	1.25	2.66	0.86		—3.39
21	Lactalbumin	3.19	0.77	2.77	0.14	2.45	2.88	3.34	0.68	—0.79	
22		3.33	1.05	2.72	0.71	2.64	0.45	2.98	0.62	—0.36	
23		3.79	1.42	3.00	0.55	2.40	2.95	3.15	1.05	—1.31	
24		3.42	0.83	2.66	1.07	2.94	0.65	3.22	1.24	—0.74	
Period (6)	Av.	3.43	1.02	2.79	0.62	2.61	1.73	3.17	0.90		—0.80
25		3.18	1.66	2.46	0.24	2.44	1.89	2.45	0.59	—3.09	
26		3.24	1.79	2.65	1.47	2.47	0.77	2.37	0.55	—3.19	
27		2.70	0.72	2.21	0.32	2.38	0.66	2.53	1.01	—2.49	
28		2.69	0.89	2.09	0.05	2.41	0.77	2.77	0.79	—2.55	
Period (7)	Av.	2.95	1.27	2.35	0.52	2.43	1.02	2.53	0.74		—2.83
29	Casein	2.84	0.98	2.18	0.65	3.20	0.96	2.36	0.88	—0.24	
30		3.20	1.52	2.81	0.30	3.19	3.04	2.56	0.58	—1.04	
31		3.49	1.27	2.61	0.25	3.39	0.15	2.84	1.38	—0.58	
32		3.95	0.91	2.84	1.44	3.25	0.91	2.58	0.67	—0.87	
Period (8)	Av.	3.37	1.17	2.61	0.66	3.26	1.27	2.59	0.88		—0.68
33		3.84	2.03	2.51	0.20	1	1	2.14	0.50	—3.13	
34		3.51	1.30	2.78	0.32	1	1	2.06	0.88	—3.00	
35		3.20	1.16	2.10	0.32	1	1	1.88	0.38	—2.41	
36		3.56	0.69	2.25	0.21	1	1	2.56	1.02	—2.82	
Period (9)	Av.	3.53	1.29	2.41	0.26			2.16	0.70		—2.84
37	Lactalbumin	3.53	1.27	2.46	1.36			2.58	0.90	—0.90	
38		3.75	1.57	2.39	0.36			2.51	0.72	—0.63	
39		4.12	1.75	2.23	0.57			2.54	0.82	—0.87	
40		4.17	0.80	2.34	0.26			2.64	0.59	—0.46	
Period (10)	Av.	3.89	1.35	2.36	0.64			2.57	0.76		—0.71

<sup>1</sup> Data omitted because subject contracted "influenza" on thirty-seventh day. Average balance for the 4 days was —2.99 gm.

have been expected. Maximum variation is seen in period 6 (lactalbumin) and period 8 (casein). Even these daily variations, however, are slight. When the data are averaged for each subject, the similarity in performance of the 4 men is even more apparent (table 3). A comparison of the individual balances during the supplemented periods shows that during both casein periods the 4 subjects had very uniform values, but that both lactalbumin periods were characterized by somewhat more variation between individuals. None of the variations were sufficiently large to change the interpretation. The uniformity between individuals under the conditions of this experiment is suggested by the so-called "endogenous" values during the 4 depletion periods. The maximum variations shown by individuals during these periods were only 0.65, 0.78, 1.00 and 0.66 gm (table 3) of nitrogen. This observation is of value in indicating that the subjects were in a steady state of nitrogen metabolism and hence suitable for comparative assay.

The average balances of the 4 subjects are presented in figure 1 by means of the method of graphic presentation suggested by Reifstein, Albright and Wells ('45). The nitrogen intake is plotted as an area from the zero line toward the bottom of the diagram; the total excretion is indicated by the height of the column above the intake level, and the urinary and fecal values are indicated by the waved line. In this arrangement the projections of the columns above the zero line represent negative balances. A positive balance is shown by that amount by which the total height of the column does not reach the zero line. Since all balances in this study were negative, the differences in the height of the columns above the zero line are of major interest. The most pertinent portion of the columns have been darkened merely for emphasis.

One can see by inspection that the balances with casein and lactalbumin are identical for all practical purposes. Both fecal and urinary excretions show exactly the same pattern of changes with both proteins. There is 1 exception, namely, the high urinary nitrogen during the first depletion period follow-



ing casein supplementation. This may be partially explained on the basis of a transient infection shown by 1 subject who experienced loose stools, otorrhea and rhinitis on this day. One other subject on this day reported that he was developing a cold. This explanation is probably valid since the depletion period following the administration of casein for a second time gave average excretion values consistent with those following other supplementation periods.

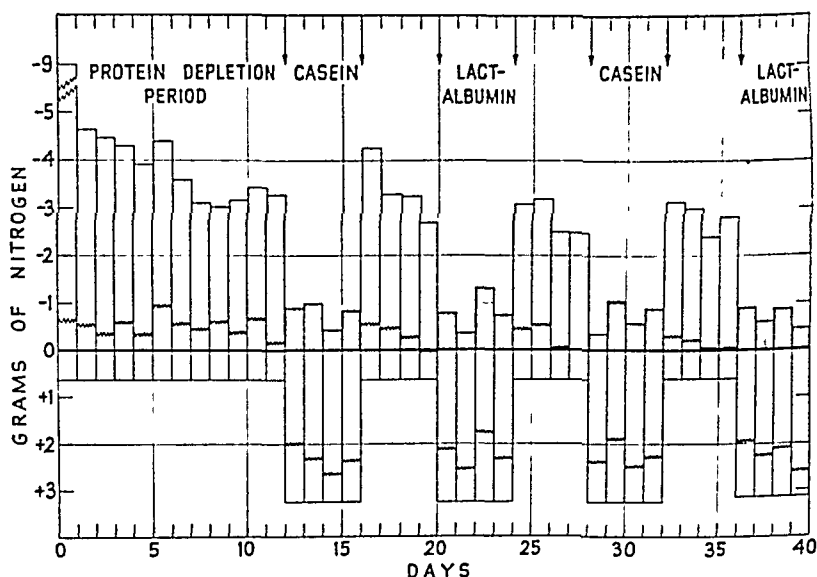


Fig. 1 Average daily nitrogen intake, nitrogen balance and urinary and fecal nitrogen excretion of 4 healthy men during protein depletion and subsequent supplementation with casein and with lactalbumin. Protein was added to the basal diet (0.008 gm nitrogen per kg) to supply 0.033 gm N per kg body weight. See text for construction of the figure.

The figure clearly indicates also that the fecal excretion during the depletion periods was always greater than the nitrogen intake, strongly suggesting that much or all of the nitrogen of the basal diet was not available for utilization.

Table 3 gives the calculated biological values. We have employed the method of Mitchell ('24), and for the individual calculations used the average endogenous urinary and fecal

nitrogen of each subject for the 4 depletion periods. The values show good agreement. The maximum variation between individuals on a single assay (which is also the greatest variation in the whole series) was observed in the first lactalbumin period as that between 100 and 72.3. This variation approximates that reported by Mitchell ('24, table 12) when the assay method was first described. The average biological values for the 2 casein periods were 89.0 and 94.7, and for the 2 lactalbumin periods 90.2 and 93.3. With the variables known to exist in this type of assay, these values must be regarded as practically identical.

TABLE 3

*Nitrogen balances during depletion and supplementation, and biological values.*

SUBJECT	A	B	C	D	AVERAGE B. V. <sup>1</sup>
All nitrogen values are in grams per day					
Depletion (9-12 day)	-3.92	-2.26	-3.81	-2.90	
Casein	-0.76	-0.64	-0.83	-0.93	
B. V.	(94.5)	(88.5)	(84.6)	(88.4)	89.0
Depletion (17-20 day)	-4.13	-2.88	-3.52	-3.00	
Lactalbumin	-0.65	-0.47	-0.69	-1.40	
B. V.	(97.4)	(91.2)	(100)	(72.3)	90.2
Depletion (25-28 day)	-3.48	-2.29	-2.81	-2.75	
Casein	-0.74	-0.32	-0.57	-0.79	
B. V.	(98.9)	(97.8)	(87.3)	(94.7)	94.7
Depletion (33-36 day)	-4.09	-2.10		-2.34	
Lactalbumin	-1.44	-0.05		-0.66	
B. V.	(84.4)	(100)		(95.5)	93.3
Av. depletion	-3.91	-2.38	-3.38	-2.75	

<sup>1</sup> Biological value.

### DISCUSSION

Early observations (Osborne and Mendel, '15) indicated that casein and lactalbumin were nutritively different when measured by the growth of rats, and this finding was subsequently extended to include dogs (Lewis, '17), mice (Bauer and Berg, '43) and chicks (Almquist, '42). It was only natural to interpret these animal findings in terms of human nutrition,

since there is a preponderance of casein in cow's milk and the lactalbumin content of mother's milk is higher than its casein content. Since clinical experience indicated that better results attended the feeding of infants with mother's milk, it was logical to attribute part of the superiority to a higher content of lactalbumin (Holt, '16).

The nutritive value of the 2 milk proteins in the feeding of infants was first put to experimental test by Edelstein and Langstein ('19). These investigators reported nitrogen balance studies on 4 infants who received varying levels of either casein or lactalbumin for various periods. Their one best experiment was with an 11-week-old child who was given a protein-free diet (cane sugar, carrots, starch, butter and salt solution) for 12 days, followed by an 18-day period on casein, a 6-day protein-free period, and a final 13-day lactalbumin period. We have recalculated their data to obtain biological values comparable with those reported here: for casein the value was 85.0; for lactalbumin, 87.4. During the 18-day casein period, the infant gained 500 gm in weight; during the lactalbumin period, 190 gm. Two additional infants, observed only for single 3-day periods, had recalculated biological values for casein of 77.4 and 65.3, and for lactalbumin of 98.2 and 67.0, respectively. Observations on the fourth infant were so interrupted by technical difficulties as to be uninterpretable. The authors concluded that lactalbumin was superior to casein for infant nutrition. Our study of their data in which we made no selection of values, does not support such an interpretation. With consideration of the difficulties of working with infants and of the variation in biological values even in adults under very uniform conditions, their data do not demonstrate to us any significant difference in the 2 proteins in infants.

It was not until Harrison ('36) showed that adequate nitrogen retention could be attained by feeding cow's milk proteins to infants at a low intake level, and Gordon, Levine, Wheatley and Marples ('37) showed that similar nitrogen retention in premature infants could be attained by feeding equal levels of nitrogen as cow's milk and as mother's milk that the earlier

concepts of the different nutritive qualities of the 2 proteins for man became less tenable.

It has been repeatedly demonstrated that the nutritive value of casein for rats or dogs can be made equal to that of lactalbumin by the addition of a small amount of cystine or methionine. Using a casein hydrolysate supplemented with methionine, Cox and associates ('47) showed that no effective improvement in nitrogen retention resulted from the added methionine when the hydrolysate alone or supplemented with this amino acid was administered to 4 groups of persons of varying protein need: (1) infants; (2) adults on a maintenance nitrogen level; (3) protein-depleted adults allowed less nitrogen than that necessary to attain nitrogen equilibrium; and (4) adults nourished solely by intravenous feedings. While this evidence strongly suggested that intact casein might be the nutritional equivalent of intact lactalbumin in man, it could not substitute for an actual experimental comparison of the 2 proteins.

The present experiment in which the 2 proteins have been compared at low intake levels in healthy but slightly protein-depleted adults, leaves no doubt that the 2 substances are of equal value for adult maintenance. It is possible that a comparison at higher intake levels would disclose some difference, but our earlier findings (Cox et al., '47) with a methionine supplemented casein hydrolysate at different intake levels would certainly indicate that this is unlikely. A new comparison of the 2 proteins should be made in infants, since the requirements of the growing child may be different from those of the adult. However, in view of the work cited above (Edelstein and Langstein, '19; Harrison, '36; Gordon et al., '37; Cox et al., '47) it is unlikely that any difference between the 2 proteins will be found even in infants.

#### SUMMARY AND CONCLUSION

Casein and lactalbumin have been fed at an intake level of 2% of the calories to 4 well adults following a 12-day protein depletion. Two 4-day periods of supplementation with each protein were alternated with 4-day depletion periods. Nitro-

gen balances and calculated biological values indicated that casein and lactalbumin are equally effective in maintaining nitrogen balance in adult man.

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# THE GROWTH-PROMOTING ACTION OF CELLULOSE IN PURIFIED DIETS FOR CHICKS<sup>1</sup>

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Studies on "crude fiber" in rations of small laboratory animals have been reported previously, but there exists in the literature much controversy as to its need and utilization by poultry. It is generally understood that excessive levels of "crude fiber" in poultry rations reduce feed efficiency, growth, and egg production. On the other hand, the presence of "crude fiber" appears to be beneficial under certain conditions. For example, Sheehy ('39) and Bearse, Miller and McCleary ('40) observed that cannibalism is prevented by the introduction of extra "crude fiber" in the ration. However, such studies have been made with practical-type poultry rations, and the fiber supplement may have contained effective nutrients other than fiber.

In the study presented here, a purified source of cellulose was added to a "synthetic" ration nutritionally complete in all the known nutrients and free of fiber. The results show that the addition of cellulose to such a ration improved the growth rate and was otherwise beneficial.

Woolley and Sprince ('45) showed that 20% of cellulose supplied as Cellu-flour or powdered cellophane in purified guinea pig rations was active as a growth stimulant. No other report on the growth promoting-action of cellulose in purified

<sup>1</sup> Scientific paper no. A165. Contribution no. 2060 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

rations for small laboratory animals (non-ruminants) is available. Workers in various laboratories have included cellulose materials in purified poultry rations but have not made any reports on its growth-promoting effect.

### EXPERIMENTAL PROCEDURE AND RESULTS

Day-old New Hampshire chicks were reared in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum* throughout the 4-week experimental period.

TABLE 1  
*Composition of basal ration 113.*

Main ingredients	%	Supplements	mg/100 gm
Glucose ("Cerclose")	61.4	Thiamine HCl	0.4
Casein (crude)	18.0	Riboflavin	0.8
Gelatin	10.0	Ca pantothenate	2.0
Soybean oil	4.0	Choline Cl	200.0
Salts 1 M (Briggs, '46)	6.0	Nicotinic acid	5.0
l(—)Cystine	0.3	Pyridoxine HCl	0.6
		Biotin	0.02
		Folic acid	0.2
		i-Inositol	100.0
		Para-aminobenzoic acid	0.2
		Alpha-tocopherol	0.5
		2-Methyl-1, 4-naphthoquinone	0.1
	Vitamin A in I.U. 1200 <sup>1</sup>		
	Vitamin D <sub>3</sub> in A.O.A.C. units 170 <sup>1</sup>		

<sup>1</sup> Vitamins A and D<sub>3</sub> are fed by dropper weekly.

The basal ration (113) given in table 1 was used in the experiments and contains all nutrients known to be required by the chick. All supplements with cellulose<sup>2</sup> were made at the expense of glucose. Feed consumption records were taken in order to obtain feed efficiency data, and the chicks were weighed individually each week.

<sup>2</sup> "Ruffex," a roughage material distributed by the Fisher Scientific Co., Pittsburgh, Pa., was used. It is processed from rice hulls and contains 70% alpha cellulose, the balance being simple and hydrocelluloses. It is reported to contain neither proteins, fats nor vitamins and gives an ash value of less than 1%.

The results of 2 series, summarized in table 2, show that the addition of from 5 to 15% of cellulose in the ration resulted in increased growth and apparently greater feed efficiency values. The greatest response in growth was at the 5% level of cellulose. The 10% and the 15% levels of the cellulose also gave better growth and feed efficiencies when compared with the basal ration. Increasing additions of the supplement through 50% resulted in decreased growth, decreased feed efficiency values, and smaller statures when compared with chicks in group 1. More recent feeding trials (not shown in the table) have given similar growth responses with cellulose.

TABLE 2

*Summary of the effect of cellulose supplements in purified chick rations.*

GROUP	SUPPLEMENT TO BASAL DIET 113	NUMBER CHICKS STARTED	NUMBER DEAD AT 4 WEEKS	AVERAGE WEIGHT AT 4 WEEKS	FEED EFFICI- ENCY <sup>1</sup>
				<i>gm</i>	
1	None	16	2	308	0.491
2	5% Cellulose	16	0	354	0.587
3	10% Cellulose	16	0	340	0.560
4	15% Cellulose	16	0	340	0.564
5	20% Cellulose	16	0	288	0.434
6	30% Cellulose	12	0	268	0.311
7	40% Cellulose	12	0	200	0.312
8	50% Cellulose	12	1	140	0.215

<sup>1</sup>  $\frac{\text{Total gain in weight}}{\text{Total feed intake}}$

No difference in feathering and no feather picking or cannibalism were observed in any of the experimental groups. It is interesting to note that as high as 50% of cellulose was consumed with practically no mortality, although growth was poor. As much as 15% of cellulose in the ration was tolerated without any ill effects. Statistical treatment given in table 3 showed that the 5%, 10% and 15% supplemental levels gave significantly greater growth responses compared with group 1.

The droppings of the chicks on all levels of cellulose supplements were of a drier, firmer consistency than those of the controls. Feed consumption values and chemical analysis of



the droppings for a 1-week period gave digestibilities of 53.9%, 27.9%, and 34.3% for the "crude fiber" fed at the 5%, 10% and 15% levels of cellulose, respectively. Analysis of the cellulose for "crude fiber" gave a value of 75.38%.<sup>3</sup>

TABLE 3  
*Analysis of variance.*

SOURCE OF VARIATION	D.F.	VARIANCE
Treatment	3	5502.33 <sup>1</sup>
Error	58	1796.59

<sup>1</sup> Significant to 0.05 level.

### DISCUSSION

The exact reason for the increased growth obtained by feeding cellulose is as yet unknown, but it is believed to be due to the presence of cellulose itself rather than to a possible contaminant (unpublished data). It is possible that hydrolysis of the alpha cellulose and of the simple and hydro-celluloses in the intestinal tract gives rise to small amounts of growth stimulatory product(s) other than glucose or other than products that may be derived from glucose. Enzymes of microbiological or intestinal origin, or both, may be active in such hydrolyses. It is known that certain microorganisms produce cellulose-splitting enzymes. In addition, Baker ('42) and Hungate ('44, '46) have shown that various microorganisms possess the ability to metabolize cellulose. One or more of the decomposition products derived from cellulose metabolism may act as growth factors.

It is also possible that the mere physical presence of the cellulose may be beneficial in some manner. For instance, its presence in restricted amounts could aid in the intestinal absorption of the nutrients. However, the feed efficiency values for the 5%, 10% and 15% levels of cellulose given in table 2 show that cellulose is probably not functioning simply as a bulk factor.

<sup>3</sup> We wish to thank Robert G. Fuerst for these analyses

The retarded growth and lowered feed efficiency values with the feeding of the 20% through 50% levels of cellulose were probably caused by a decrease in the availability of metabolizable simple carbohydrates, since the supplements were fed at the expense of glucose.

This work shows the importance of including some source of cellulose (or perhaps its decomposition products) in purified laboratory rations in order to obtain maximum growth and feed efficiency. In addition, the beneficial effect of limited amounts of fiber in practical rations may be more easily studied and better understood in view of this finding.

#### SUMMARY

The addition of 5%, 10% and 15% of cellulose to a purified chick diet free of fiber but complete in all known nutrients resulted in a significant increase in growth. Additions of cellulose at levels ranging from 20% through 50% resulted in retarded growth but there was practically no mortality in these groups under the experimental conditions described. Possible reasons for the growth-promoting effect of cellulose are discussed.

#### ACKNOWLEDGMENTS

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# A STUDY OF THE FASTING-HOUR EXCRETION OF THIAMINE IN THE URINE OF NORMAL SUBJECTS<sup>1</sup>

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## ONE FIGURE

(Received for publication May 16, 1947)

A number of excretion tests have been used as a measure of nutritional status with respect to thiamine. All are based upon the premise that the amount of thiamine available for excretion by the kidney depends more or less upon the adequacy of the supply in the body (Melnick, '42). Of all the procedures employed the fasting-hour excretion test, first recommended by Holt and Najjar ('42), appears to be the most feasible for nutritional surveys.

Melnick and Field ('42) reported good correlation between 24-hour output and fasting excretion of thiamine in 37 subjects. The fasting specimens in this study, however, represented a 4-hour period. Oldham and her coworkers ('44) in their study of the thiamine requirement with 2 young subjects of pre-school age concluded that the 1-hour fasting excretion test reflected the nutritional status with respect to thiamine. To our knowledge no one has reported any extensive comparison of the 24-hour urinary excretion of this vitamin with that in a 1-hour specimen collected immediately after the completion of the 24-hour period and following an overnight fast. Najjar and Holt ('43) apparently accumulated such data but we have not seen these published. Since the daily

<sup>1</sup> Aided by grants from the Nutrition Foundation, Inc., and from the University Center in Georgia.

urinary output of thiamine has been used widely in assessing thiamine nutritional status (Allen, '43; Elsom et al., '42; Jolliffe et al., '39; Melnick and Field, '42), it seemed to us worthwhile to compare this value with that of a fasting 1-hour specimen collected in the same metabolic period by normal individuals. This paper presents such values obtained from 63 young adults. In an attempt to more properly evaluate the fasting-hour excretion test with the view of its application to survey work, a 4-hour clearance test following an oral test dose of 1 mg of thiamine hydrochloride was also made on 20 of the subjects.

#### PROCEDURE AND METHODS

The subjects were 59 young men and 4 young women between the ages of 19 and 28. They were all university students, and none had apparent signs of any vitamin deficiency. No attempt was made to control the diet since the 24-hour specimen and the subsequent fasting-hour specimen from each individual were both collected during the same metabolic period. The 24-hour urine collections were made in the conventional manner, beginning after the voiding of the morning specimen of 1 day and ending with the morning urine of the following day. The fasting-hour urines were collected essentially according to the procedure recommended by Holt and Najjar ('42). The subjects were instructed to abstain from food or beverages except water beginning with the fourteenth hour of the 24-hour period. Upon completion of the 24-hour collection they were asked to note the time and drink 1 glass of water. The fast was continued for another hour at the end of which the fasting specimen was collected. This urine sample thus represented the hourly output at the end of a fast of 11-13 hours' duration. Where the fasting specimen represented more or less than 60 minutes, the time of voiding was recorded and the hourly fasting urinary output was calculated.

In the clearance tests, the subjects came to the laboratory before the completion of the fasting hour, that is, the twenty-

fifth hour of the total period. When this hour was completed and the fasting specimen had been collected, the subjects were given 1 mg of thiamine hydrochloride in solution by mouth. All the urine voided during the next 4 hours was collected and analyzed for urinary thiamine. During this clearance period only water and black coffee were allowed.

The specimens were preserved with glacial acetic acid (2-3 ml per 100 ml) and stored in dark bottles in the refrigerator until analysis for thiamine was made. The analyses were all completed within 5 weeks of the day of collection. We have been able to verify the statement of Egaña and Meiklejohn ('41) that the thiamine content of urine thus preserved remains unaltered for more than 6 weeks.

The thiamine content of the specimens collected by the first 43 subjects was determined by the Merck and Company, Inc., adaptation ('41) of the Hennessy and Cerecedo thiochrome method (Hennessy, '41). Calculations, however, were made according to the formula of Najjar and Ketron ('44) which is believed to correct for the reduction of fluorescence caused by oxidation of  $F_2(N^1\text{-methylnicotinamide})$ . The specimens of the last 20 subjects were analyzed by a fluorometric adaptation (Papageorge and Lamar, '47) of the Urban and Goldman ('44) thiochrome method which employs benzenesulfonyl chloride for the destruction of thiamine in the blank determination.

## RESULTS

Figure 1 presents the comparison of the 24-hour with that of the fasting 1-hour excretion of urinary thiamine in the 63 subjects studied. The scatter diagram shows that there is a definite correlation between the 2 values, particularly at lower levels of excretion. Assuming a linear relationship between the 1-hour fasting excretion of thiamine and the 24-hour output, the coefficient of correlation<sup>2</sup> is 0.70. This

<sup>2</sup> Coefficient of correlation was calculated according to the equation

$$r_{xy} = \frac{N\sum XY - (\sum X)(\sum Y)}{\sqrt{[N\sum X^2 - (\sum X)^2][N\sum Y^2 - (\sum Y)^2]}}$$

Guilford, J. P. ('42) *Fundamental Statistics in Psychology and Education*, p. 204. McGraw-Hill Co., New York.

indicates good correlation. The 11 points representing the lowest 24-hour and fasting 1-hour excretion values observed all fall within the 2 regression lines.<sup>3</sup>

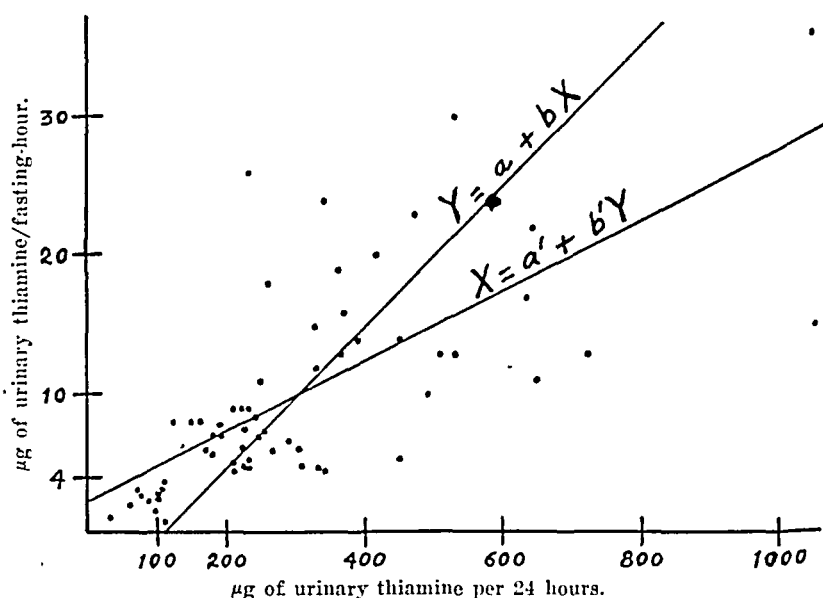


Fig. 1 Correlation of 24-hour with fasting-hour excretion of thiamine in the urine of 63 subjects. Coefficient of correlation is 0.70. Intersection of regression lines indicates mean values.

Table 1 gives the minimum, maximum and mean values of the whole series. From the mean values as well as from examination of figure 1 it is seen that the 24-hour excretion is usually more than 24 times the value of the fasting 1-hour excretion. This is not surprising since the intake of thiamine during the period of collection would be expected to influence the 24-hour output to a greater degree than it would affect the excretion after 12 hours of fasting. However, in 14 of the

<sup>3</sup>Regression lines are lines of trend along which lie the best predictions of 1 value from the other. They intersect at the point representing the mean values of the 2 variables. Since the slopes of the lines depend upon the coefficient of correlation, the higher this coefficient, the closer together will a pair of regression lines tend to lie. See Guilford, pp. 211 ff.

subjects the 24-hour value was less than 24 times that of the subsequent fasting-hour excretion of thiamine. All of these subjects excreted more than 120  $\mu\text{g}$  of thiamine per day and thus over 5  $\mu\text{g}$  per fasting-hour.

Table 2 presents the results found on the last 20 subjects on whom a clearance test was also made. Urine volumes are included in the table since they may have some bearing on the explanation of certain of the discrepancies observed between the 3 tests of thiamine status. For some reason the fasting-

TABLE 1

*Minimum, maximum and mean values of urinary thiamine excreted per day and per subsequent fasting-hour by 63 subjects.*

	THIAMINE EXCRETION	
	$\mu\text{g}/24 \text{ hrs.}$	$\mu\text{g}/\text{fasting-hour}$
Minimum 24-hr. value	33	1.1
Minimum fasting-hour value	109	0.7
Maximum 24-hr. value	1050	15.0
Maximum fasting-hour value	1044	36.0
Means of 63 values	309	10.1

hour volumes of these last 20 subjects were on the whole much smaller than those of the first 43 individuals studied. The correlation coefficients for the thiamine excretion values in table 2 are as follows: 0.80 for correlation of fasting-hour with 24-hour output; 0.64 for correlation of percentage of test dose excreted in 4 hours with amount of thiamine excreted during the fasting-hour; 0.77 for correlation of percentage of test dose return with 24-hour output of the vitamin.

#### DISCUSSION

Assuming that the 24-hour excretion of thiamine is a fair index of nutritional status with respect to this vitamin, our results indicate that the fasting-hour excretion test can be employed in assessing thiamine status where a large number of people are to be tested. This conclusion agrees with the findings of Melnick and Field ('42) on 4-hour specimens and with the statement of Oldham et al. ('44) based upon the



indicates good correlation. The 11 points representing the lowest 24-hour and fasting 1-hour excretion values observed all fall within the 2 regression lines.<sup>3</sup>

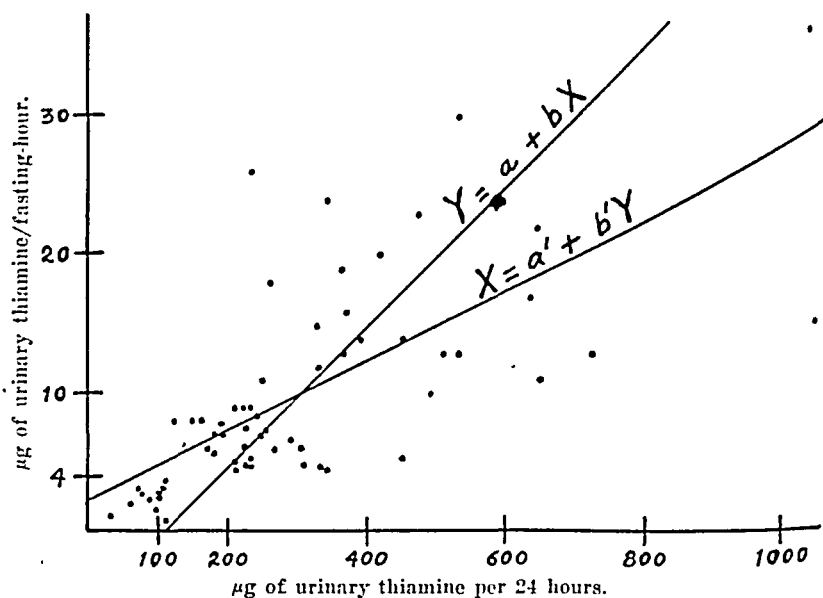


Fig. 1 Correlation of 24-hour with fasting-hour excretion of thiamine in the urine of 63 subjects. Coefficient of correlation is 0.70. Intersection of regression lines indicates mean values.

Table 1 gives the minimum, maximum and mean values of the whole series. From the mean values as well as from examination of figure 1 it is seen that the 24-hour excretion is usually more than 24 times the value of the fasting 1-hour excretion. This is not surprising since the intake of thiamine during the period of collection would be expected to influence the 24-hour output to a greater degree than it would affect the excretion after 12 hours of fasting. However, in 14 of the

<sup>3</sup>Regression lines are lines of trend along which lie the best predictions of 1 value from the other. They intersect at the point representing the mean values of the 2 variables. Since the slopes of the lines depend upon the coefficient of correlation, the higher this coefficient, the closer together will a pair of regression lines tend to lie. See Guilford, pp. 211 ff.

in subjects 53, 59 and 63, there may not have been a delay in excretion of thiamine; or, if in cases like that of subject 56 the relatively high return of the test dose may not have been due to diuresis since the clearance specimen of this individual was greater in volume than her total 24-hour collection. The completeness with which the bladder is emptied may also be a factor in determining the accuracy of results representing short periods of urinary excretion as in the case of subject 47 whose fasting-hour value was lower than one would expect on the basis of his daily output and his 4-hour test dose return.

The results of the clearance test do not throw much light on the question of the comparative value of the fasting-hour excretion and the 24-hour output as measures of thiamine status. While statistically the clearance test showed better correlation with the 24-hour than with the fasting-hour excretion, our data are open to criticism in view of the small amount of test dose (1 mg) administered orally. There is also the question of rate of excretion of vitamin in instances of extraordinarily low or extremely high urinary volumes. Giff and Hauck ('46) failed to obtain agreement in their comparison of 4 methods for studying urinary excretion of thiamine, 2 of which were essentially clearance procedures. It would seem, however, that the fasting-hour output should be less liable to fluctuations caused by sudden changes in intake than the 24-hour excretion (Holt, '43).

In attempting to arrive at a fasting-hour level of thiamine excretion which should represent the division between adequate and inadequate values, we may use established daily output as a basis. It is generally agreed that on adequate intakes the daily excretion of thiamine in urine exceeds 100  $\mu$ g and is usually around 200  $\mu$ g or more (Allen, '43; Giff and Hauck, '46; Jolliffe et al., '39; Mason and Williams, '42; Melnick and Field, '42). According to the revised daily allowances recommended by the Food and Nutrition Board of the National Research Council (Jeans, '46), the intake of our subjects, if adequate, should have been at least 1.0 to 1.5 mg

correlation of fasting 1-hour excretion with the 4-hour return of a test dose and with thiamine intake in their 2 small subjects.

To be sure, the measure of the urinary output of thiamine as an index of nutritional status is definitely limited (Elsom et al., '42). Rate of absorption and rate of excretion by the kidney are undoubtedly involved as well as level of intake and rate of utilization. While, on the whole, our data confirm the observations of others (Elsom et al., '42; Youmans et al., '40) that the amount of thiamine excreted is independent of urinary volume, there are indications that this may not hold true outside of ordinary limits. One may well ask if in instances where the fasting-hour volume is excessively small, such as

TABLE 2

*Urinary excretion of thiamine per 24 hours and per subsequent fasting hour with 1 hour urinary return of 1 mg oral test dose of thiamine in 20 subjects*

SUBJECT NO	24 HR URINE		FASTING HOUR URINE		CLEARANCE URINE	
	Vol	Thiamine	Vol	Thiamine	Vol	Return of 1 mg dose
	ml	μg	ml	μg	ml	%
44	2000	178	81	5.7	620	10.4
45	2000	192	91.5	7.8	590	9.7
46	2000	243	31	8.4	288	6.0
47	2000	450	35	5.2	500	12.4
48	2000	469	99	23.0	140	11.4
49	2200	581	151	24.0	354	15.0
50	1820	530	51.5	13.0	178	11.1
51	2000	87	31.5	2.2	130	5.1
52	2000	325	86	15.0	760	14.1
53	1500	109	14	0.7	123	3.4
54	1500	307	43.5	4.8	334	9.7
55	1000	231	37.5	5.1	187	7.1
56	790	33	35	1.1	815	7.1
57	2000	338	22.5	4.6	420	14.5
58	1000	77	57	2.9	410	4.4
59	1000	102	17	2.6	128	3.3
60	1500	106	49.5	3.0	184	3.2
61	1500	229	58	4.8	415	9.5
62	2200	224	76	4.8	149	6.8
63	1000	98	17	1.5	210	9.3
Mean						
values		245		7.01		8.6

specimen, collected in the hour following completion of the 24-hour period and after an over-night fast. The 2 values show good over-all correlation with a coefficient of 0.70.

2. The percentage of a 1-mg oral test dose of thiamine returned in 4 hours in the fasting state was also determined in 20 of the 63 subjects. The correlation between the test dose return and the 24-hour excretion was better than that between the test dose return and the fasting-hour output. However, the procedure followed in the clearance test is open to criticism.

3. The fasting-hour excretion test offers itself as a convenient method of estimating thiamine nutritional status in survey work where a large number of individuals are to be tested.

4. The critical level of fasting-hour excretion of thiamine appears to be 4  $\mu$ g. Values below this suggest a likelihood of inadequate thiamine intake.

#### ACKNOWLEDGMENTS

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of thiamine per day. Such intakes should result in 200 to 300  $\mu\text{g}$  daily excretion in the urine on the 20% basis of Melnick, Field and Robinson ('39). If we use the regression line  $Y = a + bX$  in figure 1 to get values of  $Y$  from  $X$ , the predicted fasting-hour output for the 200–300  $\mu\text{g}$  24-hour excretion is 4.5 to 9.  $\mu\text{g}$  of thiamine. The mean values for the 63 subjects are not far from these figures: 309  $\mu\text{g}$  per 24 hours and 10.1  $\mu\text{g}$  per fasting-hour.

Eleven of the subjects excreted less than 4  $\mu\text{g}$  of thiamine per fasting-hour. Seven of these had 24-hour excretions less than 100  $\mu\text{g}$  of the vitamin and the other 5 showed between 100 and 110  $\mu\text{g}$  in their 24-hour specimens. Clearance tests were made on 7 of the 11 subjects with low daily and fasting-hour thiamine excretion. Five of the 7 gave less than a 6% return of the test dose. The other 2 were subjects 56 and 63 in whom the higher test dose return may have been due to diuresis or to delayed thiamine excretion, respectively, because of their unusual urine volumes. No subject excreting over 4  $\mu\text{g}$  of thiamine during the fasting hour gave less than a 6% return of the oral test dose of 1 mg.

These data suggest that 4  $\mu\text{g}$  may be the critical level of fasting 1-hour excretion of thiamine. This value agrees with the findings of Melnick and Field ('42) who place the minimal normal level of 4-hour fasting excretion at 15  $\mu\text{g}$ . Oldham and her coworkers ('44) observed higher fasting 1-hour excretions in their 2 young subjects when the children were on an adequate allowance of thiamine. The obvious limitations of the fasting-hour excretion test as a measure of thiamine nutritional level should be borne in mind. It can be used only as an approximate index for clinical purposes and should be repeated in conjunction with a standard clearance procedure in cases of suspected thiamine deficiency. However, it offers itself as a useful tool in estimating the general trend of thiamine level in survey studies.

#### SUMMARY

1. The 24-hour urinary output of thiamine of 63 normal young adults was compared with that in a "fasting-hour"

# THE AVAILABILITY OF VITAMINS FROM YEASTS

## III. THE AVAILABILITY TO HUMAN SUBJECTS OF RIBOFLAVIN FROM FRESH AND DRIED BAKERS' YEASTS VARYING IN VIABILITY <sup>1</sup>

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ONE FIGURE

(Received for publication June 13, 1947)

In continuation of earlier investigations (Parsons and Colord, '42) in which riboflavin of yeast was found to be less available for absorption by human subjects from the fresh living cells than from non-living cells, a wider variety of yeast types have been tested. These included samples in which the riboflavin had been acquired by the yeast gradually during growth and hence was presumably inside the cell (Massock, '43) in contrast to other samples in which the greater part of the riboflavin had been added in fortification; and dried yeasts, either with dead cells only, or with a suitable degree of viability for leavening purposes. These samples gave new opportunity for observation on possible correlation of the condition of the yeast cell with the availability of its riboflavin.

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The 24-hour urinary excretions were collected in brown bottles containing acetic acid and chloroform, and a representative sample was preserved with the addition of toluol. Fecal collections were marked by the ingestion of carmine at the beginning and end of each basal and yeast period. The fecal material was preserved in acid-alcohol and assays were made

TABLE 1  
*Composition of basal ration.*

<i>Breakfast</i>		<i>Supper</i>	
	<i>gm</i>		<i>gm</i>
Tomato juice	200	Apple sauce	100
Egg, 1 medium		Milk	200
Bread, $\frac{1}{2}$ loaf		Peanut butter	25
Butter	10	Butter	10
Milk	200	Bread, $\frac{1}{2}$ loaf	
<i>Dinner</i>			
Pineapple juice or crushed pineapple	100	<i>Ingredients 1 loaf bread (in gm):</i>	
Meat loaf	100	Unfortified white flour	113
(Beef and veal 68)		Yeast F	3
(Pork 7)		Lard	4
(Egg 10)		Sugar	4
(Oatmeal or soygrits 15)		Salt	2
Cranberry relish	100	Milk (plus water as needed)	39
(Cranberries 50)		<i>Analyses conducted on aliquots of the composite diet</i>	
(Oranges 33)		Protein ( $N \times 6.25$ ) approx.	72 gm
(Sugar 17)		Thiamine	1.2 to 1.5 mg
Peas	100	Riboflavin	3.7 to 4.2 mg
Ice cream	100		
Bread, $\frac{1}{2}$ loaf			
Butter	10		
Milk	200		

from a thoroughly mixed representative sample. Collection of fecal eliminations was of necessity omitted during the experiments employing the 1-day test-dose period.

Riboflavin was determined by the fluorometric method of Conner and Straub ('41). Duplicate determinations were made on separate days for all samples. Food aliquots representing 25% or 100% of the weighed daily food intake were homogenized in a Waring Blendor and assayed for riboflavin.



## EXPERIMENTAL

Two general plans for metabolic studies were employed: 1 used previously in this laboratory in which periods of 3 to 5 days were utilized for the uniform feeding of the basal diet alone or supplemented by a given riboflavin dose; and the other devised by Melnick et al. ('45), wherein a single test-day was preceded and followed by periods of the basal diet alone. The first technic was used in the experiment which is recorded in figure 1; the second in those recorded in tables 2, 3 and 4. Riboflavin excretions for a total of approximately 360 subject-days were assayed.

The test-doses included various types of yeast as well as a positive control dose of pure riboflavin. The identities and descriptions of these yeasts are to be found in table 1 of the following paper (Kingsley and Parsons, '47). The yeasts of the series I-A, I-B, I-C and II-C were produced for the experiment in the factory and the first 3 were from 1 lot, hence yeast strain and conditions of production were ruled out as variables. Viability and moisture content were intentionally varied in the different samples: yeast I-A was a fresh raw yeast, I-B and V-B yeasts dried with little loss of viability, and I-C and II-C with entire loss of viability.

The subjects were university students, men and women, in a satisfactory nutritional state. The basal diet is listed in table 1. Uniformity of intake was sought by the use of unit lots of food: flour,<sup>3</sup> and canned peas,<sup>4</sup> tomato juice,<sup>4</sup> pineapple<sup>5</sup> and applesauce. Supplies of meat loaf, orange-cranberry relish and ice cream<sup>6</sup> were prepared and frozen at the outset of the study to be used throughout.

<sup>3</sup> Patent white flour without fortification was obtained direct from the Pillsbury Mills, Inc., at Minneapolis. Bread was baked in the laboratory 2 to 3 times per week.

<sup>4</sup> Uniform packs of peas and tomato juice were furnished through the courtesy of the Research Department of Libby, McNeill and Libby Company.

<sup>5</sup> Thanks are due to the Pineapple Research Institute of Hawaii for the gift of the specially packed crushed pineapple and pineapple juice.

<sup>6</sup> Prepared from a special formula under the direction of Mr. L. C. Thomsen, Associate Professor of Dairy Industry, University of Wisconsin.

whole is compared with the output of subjects on a positive control dose or on live yeasts highly fortified with riboflavin. In these yeasts the vitamin addition presumably remains outside of the yeast cell inasmuch as it is returned in the urine so consistently. The details of this latter experiment will be reported elsewhere.

The riboflavin of dried dead yeast II-C, similar to I-C in all phases of manufacture except in thiamine content was found to be almost completely available (table 3). These results confirm those observed with yeast I-C.

TABLE 3

*Availability to human subjects of riboflavin of dried yeast II-C with no viable cells.*

SUBJECT	CONTROL PERIOD: 4.0 MG PURE RIBOFLAVIN IN SOLUTION			TEST PERIOD: 3.4 MG RIBOFLAVIN IN 82 GM DRIED YEAST II-C			
	Basal excretion	After control dose	Control dose excreted	Basal excretion	After test dose	Test dose excreted	Availability B <sub>2</sub> in II-C yeast
	mg/24 hr.	mg/24 hr.	%	mg/24 hr.	mg/24 hr.	%	%
D.H.	1.34	3.60	57	1.26	3.10	54	95
E.P.	0.69	2.81	53	0.75	2.59	54	102
R.N.	1.11	2.96	46	1.23	3.23	58	126
H.N.	0.77	3.06	57	1.17	2.78	47	82
Average	0.98	3.11	53	1.10	2.92	53	101

Further experiments with a commercial viable dried yeast also confirmed the results obtained with I-B. Yeast V-B, bought on the open market, was similar to yeast I-B in riboflavin content and viability, and was fed to 9 subjects, 5 women and 4 men. Urinary returns after the ingestion of 3 mg of riboflavin in the form of 63 gm of this yeast indicated that this riboflavin was only partially available as only 11-18% of the yeast test-dose was returned in the urine as compared to the 41-59% excretion of a control dose of pure riboflavin by these same subjects (table 4). This comparison indicates that not more than about 20-30% of the 3.0 mg of riboflavin in the

nutritive medium in the lower bowel, nor did they carry their content of riboflavin out of the tract with the feces.

The human bioassay technic of Melnick et al. ('45) with the simplified dosage routine was also used to re-test the availability of these yeasts. Using this method, the percentage excretion of a pure riboflavin control test-dose by both male and female subjects was found to be in good agreement with similar studies reported by these authors.

TABLE 2

*Availability to human subjects of riboflavin of raw, fresh yeast I-A.*

SUBJECT	CONTROL PERIOD: 3.0 MG PURE RIBOFLAVIN IN SOLUTION			TEST PERIOD: 2.6 MG RIBOFLAVIN IN 150 GM RAW, FRESH YEAST I-A			
	Basal excretion	After control dose	Control dose excreted	Basal excretion	After test dose	Test dose excreted	Avail- ability B <sub>2</sub> in I-A yeast
	mg/24 hr.	mg/24 hr.	%	mg/24 hr.	mg/24 hr.	%	%
F.F.	0.83	2.35	51	0.73	0.93	7	14
B.N.	0.77	2.33	52	0.70	1.06	14	27
M.M.	0.84	2.47	54	1.02	1.04	1	2
L.O.	0.88	3.19	77	1.02	1.06	2	3
D.O.	0.79	1.98	39	0.74	0.73	0	0
Average	0.82	2.46	55	0.84	0.96	5	9

A 150-gm dose of raw yeast I-A containing 2.6 mg of riboflavin released little or none of its riboflavin for absorption (table 2). The excretions ranged from 0 to 14% of the intake for the 5 subjects with an average of 5% while the urinary return from a control dose of pure riboflavin ranged from 39-77% with an average of 55%. It is not clear why the appreciable decrease in the urinary output of riboflavin on the ingestion of fresh yeast I-A recorded in figure 1 has not been observed subsequently in this or in further studies with fresh live yeasts (unpublished data) with the Melnick bioassay technic. In any event, the relatively small individual variations in riboflavin return in all experiments on live non-fortified yeasts are of little importance when this group as a

unabsorbed riboflavin and intestinal synthesis appeared to contribute to increases in fecal riboflavin.

#### ACKNOWLEDGMENTS

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# THE AVAILABILITY OF VITAMINS FROM YEASTS

## IV. THE INFLUENCE OF THE INGESTION OF FRESH AND DRIED BAKERS' YEASTS VARYING IN VIABILITY AND IN THIAMINE CONTENT ON THE AVAILABILITY OF THIAMINE TO HUMAN SUBJECTS <sup>1</sup>

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### ONE FIGURE

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Human biological assays in this laboratory and elsewhere have been summarized (Parsons et al., '45b) and extended (Hochberg et al., '45) showing that bakers' compressed yeast, when fed fresh to human subjects is a poorly utilized source of thiamine as indicated by low urinary and high fecal thiamine eliminations. There was even some evidence (Parsons et al., '45b) that the ingestion of fresh yeast might be responsible for reducing the urinary thiamine output below that of a preceding basal period thus implicating yeast as a factor in reducing the availability of food thiamine. It was desired to explore this relationship further by lengthening the period of yeast ingestion and varying the dose.

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Procedures including air-drying, boiling and treatment with alcohol which reduced or destroyed viability of the yeast cell effected a marked improvement in the release of thiamine for absorption by animals (Walker and Nelson, '33; Parsons and Collord, '42; Parsons et al., '45a). Since this implied that the availability of yeast thiamine rests more directly on the vitality of the cell than on any other factor, studies were conducted to determine the availability of thiamine to human subjects from yeasts after drying processes in which the viability of the yeast cell was retained in some samples and entirely destroyed in others.

It was also observed that fresh yeasts of high thiamine content may release a varying portion of their thiamine for human absorption (Parsons et al., '45b). Hence this effect of increasing thiamine concentration upon availability of yeast thiamine was studied further.

#### EXPERIMENTAL

Determinations are reported of the urinary thiamine excretions of 15 subjects in a series of 16 diet studies in which thiamine supplements in the form of thiamine hydrochloride or yeast were added to a controlled basal diet. The 2 types of bioassay technic used and the details of the basal diet are described in the preceding paper (Price et al., '47). The method formerly in use in this laboratory was employed for the studies recorded in figure 1 and the bioassay of Melnick for those in tables 2, 3 and 4. That results with the latter method are satisfactorily reproducible in different laboratories was attested by the fact that the 2.0-mg supplement of pure thiamine hydrochloride fed to subjects in these experiments led to a 16.7 to 20% urinary return, thus falling well within the published range.

In addition to fresh yeast F and the 3 types of dried yeasts with varying degrees of viability (yeasts II-C, V-B and L-B) 2 others were used in these experiments, in which a second effect, that of increasing thiamine concentrations in the yeasts upon yeast thiamine availability, was studied. Yeast II-Z<sub>3</sub>

and III-Z<sub>3</sub> were high vitamin yeasts containing 1.0 mg of thiamine per gm on a dry basis, or approximately 50-70 times the amount found in ordinary compressed bakers' yeast. They are of further interest since the thiamine content of the yeasts was attained by 2 different methods. The high content of yeast II-Z<sub>3</sub> was reached by synthesis and absorption of thiamine by the yeast itself during propagation in a nutrient medium. In the case of yeast III-Z<sub>3</sub> only a small percentage (approximately 2%) of the thiamine was attained by the yeast itself. The remainder of the thiamine was added in the form of thiamine hydrochloride to the thick suspension of resting

TABLE 1

*Characterization of the baking yeasts tested for their influence on availability of thiamine and riboflavin.*

IDENTITY OF THE YEASTS	CONTENT, DRY BASIS		REMARKS ON TYPE
	Riboflavin	Thiamine	
	$\mu\text{g/gm}$	$\mu\text{g/gm}$	
I-A <sup>1</sup>	55	19.5	Fresh compressed type. Live cells
I-A (boiled)	55	19.5	Same lot. Cells killed by heat.
I-B <sup>1</sup>	55	12.0	Same lot. Dried with retention of viability.
I-C <sup>1</sup>	55	11.8	Same lot. Dried with total loss of viability.
II-C <sup>1</sup>	47	65.5-74.3	Like I-C except high in thiamine acquired in growth.
II-Z <sub>1</sub> <sup>1</sup>		100	} High thiamine acquired during growth by biosynthesis and absorption. Live cells.
II-Z <sub>2</sub> <sup>1</sup>		500	
II-Z <sub>3</sub> <sup>1</sup>	44	1000	
III-Z <sub>1</sub> <sup>2</sup>		100	} High thiamine chiefly from fortification. Live cells.
III-Z <sub>2</sub> <sup>2</sup>		500	
III-Z <sub>3</sub> <sup>2</sup>	45	1000	
F <sup>2</sup>	55	18	Regular, fresh, for use by bakers. Live cells.
V-B <sup>2</sup>	48	10	Retail dry yeast for leavening. Live cells.
L-B <sup>2</sup>	146	68	Fortified retail dry yeast for leavening. Live cells.

<sup>1</sup> Special lots obtained directly from the manufacturer.

<sup>2</sup> Commercial products.



cells and thoroughly distributed by mechanical mixing before compression. These yeasts were fed to 5 subjects before each meal of the test day in doses equivalent to 5 mg of thiamine and urinary excretions of thiamine were compared with those obtained after subsequent test doses of 5 mg of thiamine hydrochloride. The other members of the II-Z and III-Z series in table 1 were used in *in vitro* experiments only.

Thiamine was determined by the thiochrome method of Hennessey ('42).

### RESULTS

The implication given in an earlier study (Parsons et al., '45b) of an interference with absorption of food thiamine from the ingestion of fresh yeast was fully borne out by the

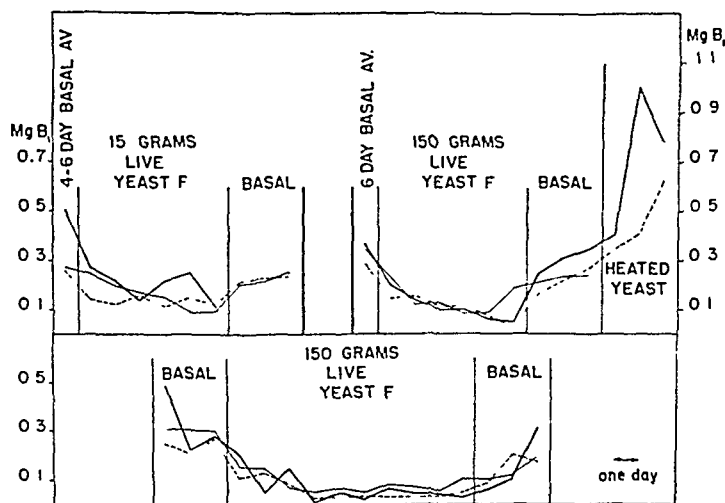


Fig. 1 The effect of ingestion of live yeast on the urinary output of thiamine.

feeding of 15 gm or 150 gm of yeast F for periods of 5 or 10 days. (See fig. 1. The averages of these data were published in a preliminary report by Ness et al., '46). The urinary output of thiamine fell below the basal level on the first day of yeast ingestion and continued to fall rapidly for about 4 days, reaching an average level of about 100  $\mu$ g output for

the 15-gm yeast dose and 50  $\mu$ g for the 150-gm dose; the minimum excretions reached as low as around 20  $\mu$ g. Within the limits of the experiment, therefore, the greater the amount of yeast fed, the greater the apparent interference with absorption of food thiamine. Somewhat the same correlation was seen in the response to the removal of the live yeast from the diet: the longer the time on the larger dose of yeast, the slower the resumption of the previous output of urinary thiamine when the basal diet alone was again fed.

These results have a theoretical interest, in that this is a new example of interference with food thiamine to be added to others reported in the literature, notably the destruction of thiamine in the digestive tract by thiaminase from certain fishes and sea foods in the diet, the thiamine deficiency in cattle resulting from the ingestion of bracken and the anti-thiamine action of certain analogues of thiamine (Wright, '47, a review).

Practical applications of the results in regard to the dietary status of live yeast are obvious. Doses of yeast larger than those recommended by the manufacturer were deliberately included in this experiment inasmuch as it seemed probable that yeast intake by the public might include a considerable range of dosage in instances where fresh yeast was consumed with the notion of using it as a supposed therapeutic agent against furunculosis or acne rather than primarily as a vehicle for a given vitamin allowance.

The results in the feeding of 3 types of dried yeast in 2 of which the viability was retained and in the other, destroyed, indicated that drying as such had little or no relationship to the effect of the yeast on the absorption of thiamine from the human digestive tract, but a correlation was evident with respect to the vitality of the yeast cell after the drying process (table 2). In 8 of the 9 tests with subjects ingesting the thiamine supplement in the form of live, dried yeasts urinary outputs were maintained at levels no higher than those of the preceding basal period which would indicate no measurable availability of yeast thiamine; in fact, in 7 of the

9 tests, the subjects responded to the dose with a decrease from the previous basal urinary level. This has been shown to be characteristic of the response to fresh compressed yeast intake. Only 1 subject, D.H., exhibited an increase in urinary thiamine excretion from 1 of the 2 yeast supplements, an amount equivalent to 1% of the yeast thiamine intake; calculated from the percentage increment following the positive control dose of 2.0 mg of thiamine hydrochloride, the physio-

TABLE 2

*Changes in urinary thiamine following ingestion of the vitamin in dried yeasts and in pure solution.*

SUBJECT	PORTION OF TEST DOSE EXCRETED				SUBJECT	PORTION OF TEST DOSE EXCRETED	
	Control dose 2.0 mg thiamine		Increments or decrements due to doses live dried yeasts			Control dose 5.0 mg thiamine	Dead, dried yeast II-C 5 mg thiamine
			Yeast L-B	Yeast V-B			
	$\mu g$	%	$\mu g$	$\mu g$		%	%
B.S.	400	20.0	—9	—15	E.P.	19.3	21.3
D.H.	395	19.7	+21	—37	D.H.	19.7	20.4
J.D.	330	16.5	—26	—75	H.N.	20.5	18.4
C.S.	425	21.3	—119		R.N.	20.9	23.0
Z.A.	360	18.0	0				
A.W.				—32			
Average		19.1				20.1	20.8

logical availability of this yeast to D.H. was roughly 7% which fell far short of the full availability claimed by the manufacturer for this type of yeast.

Regardless of the amount of live yeast ingested in these experiments none of the subjects experienced diarrhea. Laxation rate as measured by the appearance of carmine markers in fecal eliminations (Parsons et al., '44) was not uniformly affected by the ingestion of live yeast; evacuation time in those experiments ranged from 13 to 97 hours with no apparent correlation with the amount of urinary thiamine output.

In contrast to the results with live dried yeast, observation of 4 subjects ingesting 5.0 mg of thiamine in the form of a nutritional type of dried yeast, II-C, in which the yeast cells

were dead indicated full availability of the yeast thiamine. In all cases the increase in urinary thiamine output which followed the ingestion of this yeast fell within the range of from 19 to 23% excretion of yeast thiamine comparable to that obtained from the 5.0-mg control dose given these same subjects thus indicating that thiamine was fully available from this dead, dried yeast.

Data in tables 3 and 4 indicate the effect of increasing thiamine concentrations upon the availability of yeast thiamine. Yeast series II-Z and III-Z differed in that the content

TABLE 3

*In vitro* release of self-synthesized and mechanically added thiamine from yeasts.

IDENTITY OF YEASTS		THIAMINE CONTENT	THIAMINE FOUND PER ENTIRE SAMPLE					
			In solution		In yeast residue		Total	
		mg/dry gm	mg/dry gm	%	mg/dry gm	%	mg/dry gm	%
II-Z								
Thiamine synthesized or absorbed during propagation	II-Z <sub>1</sub>	100	1.0	1.0	98	98	99	99
	II-Z <sub>2</sub>	500	5.0	1.0	495	99	500	100
	II-Z <sub>3</sub>	1000	40.0	4.0	940	94	980	98
III-Z								
90% or more of thiamine added after propagation	III-Z <sub>1</sub>	100	43	43	65	65	108	108
	III-Z <sub>2</sub>	500	330	66	170	34	500	100
	III-Z <sub>3</sub>	1000	830	83	170	17	1000	100

of yeast series II-Z was attained by synthesis and absorption during propagation whereas in the case of III-Z thiamine was added mechanically to a suspension of mature yeast cells immediately before compression. As a precaution, yeast series III-Z was maintained at very cool temperatures throughout the fortification process and until the yeasts were tested *in vitro* or eaten by the subjects. By such a method of preparation it was believed that the greater part of the added thiamine might not be removed from the suspension medium by the live yeast cell.

*In vitro* studies (table 3) showed a more striking difference than did human bioassays (table 4) in the effect of the 2 methods of thiamine attainment upon its release by the yeast cell. Assays of thiamine released *in vitro* into supernatant solutions and that held by the yeast residue were conducted on both types of yeast with graduated thiamine content, following 2 minutes of shaking and 10 minutes of centrifugation

TABLE 4

*Urinary excretion of thiamine following ingestion of vitamin in fresh yeasts II-Z<sub>2</sub> and III-Z<sub>2</sub> and in pure solution.*

SUBJECT	CONTROL DOSE 5 MG THIAMINE HYDROCHLORIDE				Yeast II Z <sub>2</sub> 5 MG THIAMINE		Yeast III Z <sub>2</sub> 5 MG THIAMINE	
	Urinary thiamine /24 hrs			Return of test dose <sup>1</sup>	Return of test dose <sup>1</sup>	Avail- ability <sup>1</sup>	Return of test dose	Avail- ability
	Basal day	Test day	"Lag" day					
	mg	mg	mg	%	%	%	%	%
SS	0.27	0.83	0.40	14.0	2.4	17.1	4.2	30.0
ED	0.26	1.10	0.39	19.4	2.0	10.3	2.8	14.4
PG	0.27	1.10	0.32	17.6	2.4	13.6	1.8	10.2
BS	0.29	1.15	0.44	20.2	5.8	28.7	4.6	22.7
JO	0.17	0.91	0.42	19.8	5.2	26.3	10.8	54.5
Average	0.25	1.02	0.39	18.2	3.5	19.2	4.8	26.4

<sup>1</sup> Urinary increment from dose = ( $\mu$ g urinary output of vitamin on test day —  $\mu$ g output on basal day) + (urinary output on "lag" day — output on basal day).

Percentage return of vitamin in dose =  $\frac{\text{urinary increment from dose}}{\text{amount of test dose}} \times 100.$

Percentage availability =  $\frac{\text{percentage return of test dose}}{\text{percentage return of dose pure vitamin in solution}} \times 100.$

in an acetate buffer solution at pH 4 similar to the one used by Hochberg et al. ('45). These *in vitro* results indicated that mechanically added thiamine was much less firmly held by the cell inasmuch as 42% was released into the supernatant fluid, at the lowest level, increasing to 83% as the thiamine content of the yeast increased. In contrast the yeast which had attained its high thiamine content by synthesis and absorption during propagation released a maximum of only 4% of its thiamine into the supernatant layer. Human bioassays of thiamine availability from these 2 yeasts (table 4)

differed in the same direction as the supernatant concentrations, but the difference was much less than would have been expected from the *in vitro* results. The average 3.6% excretion of thiamine from yeast II-Z<sub>3</sub> and the 4.8% excretion of thiamine from yeast III-Z<sub>3</sub> are not far apart particularly in view of the large individual variations while both differ significantly from the average excretion of 18.1% following the ingestion of pure thiamine hydrochloride by the same 5 subjects. A similar divergence between the biological and *in vitro* release of thiamine from a high vitamin yeast has been reported by Hochberg et al. ('45). Whereas these workers found an *in vitro* release of 48-72% of thiamine in their high vitamin yeast, physiological availability was found to be only 17%.

The explanation of this evidence of differential release of yeast thiamine during *in vivo* and *in vitro* studies may be essentially the same as for the variation in the effects of different methods of drying of the yeast: i.e., a correlation with the activity of the yeast cell. It might be questioned whether under the less favorable conditions of the acetate-buffer medium in the *in vitro* experiments, metabolic activity of the yeast cell was sufficiently normal to permit a characteristic uptake of thiamine. On the other hand, a more favorable medium for active absorption of thiamine by the live yeast cell than the buffered solution used is apparently furnished either by fermenting wort, judged by the fate of thiamine in the brewing process (Levine, '41) or by the chyme of the upper digestive tract, as far as the evidence of reduced urinary return of thiamine when live yeast is eaten, is valid.

The mechanism for the inhibition of the availability of food thiamine is not known, but appears to be a competitive absorption of thiamine into the yeast cell in the digestive tract of the host. Other possibilities are being evaluated (Parsons et al., '47).

#### SUMMARY

Thiamine in an unfortified fresh bakers' yeast ingested by human subjects was unavailable for absorption. Yeasts high

in thiamine content, either from propagation or from fortification yielded only a small proportion of their thiamine for absorption; in the case of the highly fortified yeast a much larger proportion of thiamine was released *in vitro* in comparison.

The ingestion of a fresh unfortified bakers' yeast as a supplement to a weighed basal diet lowered the urinary output of thiamine and therefore apparently interfered with the absorption of food thiamine, this effect being greater on the larger yeast dose of 150 gm per day. The duration of the period of ingestion of fresh yeast affected the speed with which the previous level of urinary output of thiamine was again attained when yeast was removed from the diet, indicating a progressive depletion of body stores from ingestion of fresh yeast.

The drying of bakers' yeast did not of itself influence significantly the effect of the yeast on thiamine absorption; live dried yeast suitable for leavening also withheld its thiamine from absorption and interfered with the absorption of food thiamine judged by decreases in urinary output. On the other hand, bakers' yeast killed by commercial drying processes or by treatment in boiling water released its thiamine for absorption and offered no interference to absorption of food thiamine.

It is believed that live yeast cells in the digestive tract compete with the host for thiamine that is present.

#### ACKNOWLEDGMENTS

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# A STUDY OF THE HEMORRHAGIC-KIDNEY SYNDROME OF CHOLINE DEFICIENCY : THE PROTECTIVE EFFECT OF STARCH

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Griffith and Wade ('39) observed a dramatic series of events occurring in young rats 6 to 8 days after they were placed on diets deficient in choline. Necrosis, congestion and sometimes hemorrhages involving the cortical portions of the kidneys occurred, producing swollen, tense, "hemorrhagic" kidneys. Hemorrhages also occurred less frequently in the eyes, and occasionally in other organs. The syndrome was fatal in a large percentage of the animals, presumably due principally to the renal insufficiency which ensued (Griffith and Mulford, '41b). However, the animals which survived the critical period of 2 to 4 days after onset of illness showed rapid recovery, even on the same deficient diet, and the hemorrhagic appearance of the kidneys disappeared entirely within a few days, in spite of the progression of the fatty changes in the livers (Griffith, '40b). The syndrome occurred most constantly in male animals under 30 days of age.

The addition of choline to the diet completely prevented the syndrome (Griffith and Wade, '39). Methionine (Griffith and Wade, '40) and betaine (Griffith and Mulford, '41a) were also effective, and it was presumed that this was explained by the utilization of methyl groups of these substances in the synthesis of choline by the process of transmethylation described by du Vigneaud et al. ('39, '41).

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Various vitamin B factors, and vitamins K, C, and P were ineffective against the lesions produced (Griffith and Mulford, '41b). The syndrome could be prevented by limitation of the food intake. Increasing the casein level of the diet to about 25% increased the incidence, perhaps due to the more rapid growth, but above this level there was a rapid decline in incidence (Griffith and Wade, '40), supposedly due to the increased amount of methionine (above that required as methionine and cystine) available for choline synthesis (Griffith, '41a). Cystine, cholesterol and fat increased the lesions (Griffith, '40a). Unilateral nephrectomy in adult rats on a choline-deficient diet caused the syndrome to develop in the remaining kidney during the period of hypertrophy (Handler, '46). Renal decapsulation or administration of atropine afforded protection in young rats, suggesting that the tubular necrosis is secondary to vascular changes, perhaps of neural origin (Dessau and Oleson, '47), but the effect of the procedures on food intake was not recorded.

During the course of an investigation of the liver and kidney injury produced in rats by diets containing pyridine (Baxter, '45; '46), it seemed desirable to use a diet low in choline and methionine but not so low that pathological changes would result, during the experimental period, from the diet alone. Diets containing 10-18% casein, with yeast, cod liver oil, corn starch, sucrose, lard and salt mixture, without added choline were used, and apparently were satisfactory. On these diets young rats somewhat above the most susceptible age were never observed to develop hemorrhagic kidneys. Later, however, when the starch was replaced by sucrose, some of the animals did develop hemorrhagic kidneys.

Griffith ('41b) stated, without presenting data, that starch prevented hemorrhagic kidneys, and Handler and Dubin ('46) and Handler ('46) apparently observed some degree of protection. The mechanism of this protective effect was not investigated by these authors, and no attempt was made to explain it.

In the present investigation, the effects of sucrose and corn starch were compared, in more highly purified diets than those used in the pyridine experiments, and when the incidence of illness and death due to hemorrhagic kidneys was found to be significantly lower with starch, efforts were made to determine the mechanism of this effect. There seemed to be at least 3 possible explanations: (1) a choline content of the starch sufficient to give some degree of protection, (2) the presence in the starch of 1 or more factors other than choline, capable of affording protection by forming choline or through some other action, and (3) an effect of starch itself in the intestinal tract, probably on the bacterial synthesis of choline or other substances.

#### EXPERIMENTAL

##### *Animals*

Twenty-one to 23-day-old rats (Sherman strain) were placed for several days on a stock diet and then transferred to experimental diets. Most of the animals in the various experiments weighed from 30 to 45 gm at the time the experimental diets were started. In each individual experiment, 6 to 8 animals of comparable ages and weights were run simultaneously on the different diets. Care was exercised to make comparisons only between similar groups. The animals were kept in individual cages with large-mesh screen bottoms. Approximately 250 rats were used.

##### *Diets*

The composition of the basal diet used in the experiments is shown in table 1. The diet was prepared with sucrose or corn starch as desired, and the various supplements added. Corn starch extracted by the procedures to be described, was used in some experiments. Corn starch heated in an oven at 120°C. for 4 hours, and potato starch<sup>2</sup> were each used in 1 experiment.

<sup>2</sup> Manischewitz.

All diets were given *ad libitum* and the amounts eaten recorded at intervals of 1 to 3 days.

TABLE 1  
*Composition of basal diet.*

	Casein <sup>1</sup>	%
	Hydrogenated vegetable oil	20
	Corn oil	19
	Sucrose or corn starch <sup>2</sup>	1
	Salt mixture <sup>3</sup>	56
		4
<hr/>		
Supplements per Kilogram of Diet		
	mg	mg
Thiamine chloride	20	Para-amino-benzoic acid 10
Pyridoxine	20	Biotin 0.1
Riboflavin	30	Folic acid 0.2
Ca pantothenate	50	2-methyl-1,4-naphthoquinone 1.5
Nicotinic acid	200	dl- $\alpha$ -tocopherol acetate 15.0
<hr/>		
Inositol	500	Vitamin A concentrate 60,000 U.S.P. units
Ascorbic acid	500	Vitamin D concentrate 6,000 U.S.P. units

<sup>1</sup> Vitamin test casein.

<sup>2</sup> "Argo" corn starch, Corn Products Refining Co., Argo, Ill.

<sup>3</sup> Osborne and Mendel.

### *Choline determinations*

Choline determinations were done by the reineckate method as modified by Glick ('44), with the following adaptations. Duplicate 25.0 gm samples of corn starch were mixed with equal amounts by weight of pulverized pumice stone, and 10.0 gm samples with twice the equivalent amounts of pumice. Each sample was placed in a 33 × 94 mm extraction thimble and extracted in a Soxhlet extractor connected with a 250 ml boiling flask, using a single 125 ml volume of methyl alcohol. The extractions were allowed to proceed for 24 to 36 hours. Liberation of bound choline was accomplished by hydrolysis with 50 ml of a saturated solution of barium hydroxide. Separation of the choline reineckate precipitates by filtration was performed in a cold-room. The precipitates were dissolved in acetone which was collected in colorimeter tubes, and

readings were made on a Klett-Summerson colorimeter. Known choline chloride samples were run simultaneously by adding them to 50 ml volumes of barium hydroxide and subjecting them to the same subsequent treatment as the unknowns.

### *Starch-extracts*

Fifteen-hundred-gm samples of corn starch were extracted, twice by shaking with distilled water, 3 times by percolating with methyl alcohol, and once by percolating with ether. The solvents were separated by filtration and evaporated in a stream of warm air, leaving the extracted residues. Samples of some of the residues were taken for choline analyses. In 3 experiments, the combined residues from 1500-gm samples of starch were added to 500-gm lots of the sucrose-containing diet, and in a fourth experiment, the water and first alcohol residues, and the remaining residues, were added separately to similar amounts of the sucrose diet. The starch-extract diets, therefore, should have contained any extractable principle in about 5 times the concentration that was present in the starch-containing diet, assuming that extraction was complete and that none was destroyed in the process of extraction. The effects of the starch-extract-containing diets were compared with those of sucrose and starch diets.

### *Succinylsulfathiazole (SST)*

Groups of animals were placed on diets containing sucrose, starch, starch plus 2% SST, and starch plus 2% SST plus 0.2% choline chloride, and the effects of these diets compared.

### *Examination of animals*

Animals were usually observed daily, weighed, and examined for intraocular hemorrhages. Those which died during the experimental periods were autopsied and the kidneys, livers and other organs examined grossly. Microscopic sections of livers and kidneys were examined in a few cases.

Some of the surviving animals in the early experiments and all of them in the later experiments, were autopsied after about 10 days on the diets. The animals of 1 group on the choline-deficient sucrose diet were killed at intervals during the development of the renal lesions in order to observe the early changes, and a small group on the starch diet were continued on the diet for 4 months so that the chronic effects might be noted.

### *Examination of urine and feces*

The gross characteristics of the urine and feces of all of the groups were noted, and the urine from a few animals was examined for albumin and blood. Groups of 4 adult rats each, were placed on diets containing starch, sucrose, and starch plus 2% SST, and after 4 days on the diets, collections of feces were started and continued for 10 days. Choline determinations were done on the specimens.

## RESULTS

### *Comparison of effects of sucrose and starch diets*

As summarized in table 2, the incidence of illness and death from hemorrhagic kidneys was considerably greater on the

TABLE 2<sup>1</sup>

*Comparison of effects of diets containing sucrose and starch.*

DIET	NUMBER OF ANIMALS	ILL WITH LOSS OF WEIGHT <sup>2</sup>	DEAD WITH HEMORRHAGIC KIDNEYS	AVERAGE DAILY WT. GAIN FIRST 5 DAYS <sup>3</sup>
		%	%	gm
Sucrose	50	86	62	2.0
Corn starch <sup>4</sup>	44	43	16	2.7

<sup>1</sup> This table includes some of the animals of other tables.

<sup>2</sup> In some of the early experiments, the surviving animals were not autopsied and the exact incidence of hemorrhagic kidneys was not known, but these figures represent the approximate incidence.

<sup>3</sup> In the occasional animal which became ill or lost weight during the fifth day, the average gain during the first 4 days was used in calculating these figures.

<sup>4</sup> In some experiments extracted corn starch was used, while in others starch was employed without extraction. The protective effect was not decreased by extraction.

sucrose diet than on the same diet containing corn starch. The animals usually became ill a day or 2 earlier on the sucrose diet. Food intake and growth were greater on the starch diet, so that the protection was not due to limitation of food intake.

Potato starch was used in place of corn starch in 1 experiment and seemed to be effective in preventing hemorrhagic kidneys. However, so much of the starch passed through the animals undigested, that growth was poor in spite of much larger food consumption than on the corn starch. With an equal food intake, the feces from a group of animals on potato starch weighed (after drying) 7 times as much as that from a group on corn starch. While the feces from animals on corn starch were grossly similar in appearance to that on sucrose, the feces on potato starch were much more bulky, lighter in color and similar to that described in cases of refection (Bliss, '36).

#### *Choline content of corn starch*

The corn starch was found to contain 13 to 15 mg choline chloride per 100 gm.<sup>3</sup> The amount of starch used per kilogram of diet mixture therefore contained about 80 mg of choline. It was found (table 3) that the addition of 100 mg of choline chloride per kilogram of diet did not significantly decrease the incidence of hemorrhagic kidneys, though this amount of choline did increase the rate of growth of the animals (compare with table 2), and the time of development of hemorrhagic kidneys was delayed by 1 to 2 days. Two hundred mg perhaps afforded about the same degree of protection as the starch, and an occasional animal was observed to develop the syndrome on the sucrose diet to which had been added 500 mg of choline chloride per kilogram.

#### *Effects of corn-starch-extracts*

After finding that the starch contained too little choline to account for its protective effect, it then seemed that there

<sup>3</sup> The choline determinations were done on the starch as it was used in the diets, without drying. There was a loss in weight of approximately 10% on drying in an oven at 100°C.



might be present other protective factors, which would be active when extracted and added to the sucrose diet.

To test this hypothesis, starch extracts were prepared as already described. Extraction with water produced a small amount of crystalline material, probably chiefly sugars. The water retained by the starch diluted in decreasing degree the alcohols used in successive extractions. A yellow pigment was removed principally by the first alcohol extraction; a brownish material, which was insoluble in ether and acetone and was probably zein, was found principally in the second one; the latter 2 contained considerable amounts of fatty substances. The other extracts produced a pearly looking fatty material.

Choline determinations done on samples of the residues extracted from the 1500-gm lots of corn starch indicated that approximately one-third of the choline content of the starch had been extracted. This means that about 70 mg of choline were added to 500 gm of the sucrose diet by addition of the extracts.

In the first experiment comparing the effects of diets containing sucrose, starch, and sucrose plus starch-extract, the extract appeared to produce a questionably beneficial effect. However, in subsequent experiments using the extracts, the animals fared about the same or even worse than those on the sucrose diet alone (table 3), in spite of the fairly large choline content of the extracts. In one experiment using a limited number of animals, it seemed that heating the unextracted starch might have decreased its protective action. In order to rule out the destruction of an active principle by the process of extraction, 2 groups of rats were fed the diet containing extracted starch, and the survival was at least as great as with unextracted starch. It had to be concluded that the protective effect was due to an action of the starch as a whole, or possibly to substances not extracted under the conditions used.

TABLE 3  
*Effects of starch-extracts.*

DIET	NUMBER OF ANIMALS	ILL WITH LOSS OF WEIGHT	DEAD WITH HEMORRHAGIC KIDNEYS	AVERAGE DAILY WT. GAIN FIRST 5 DAYS
		%	%	gm
Sucrose + combined extract	18	72	44	2.8
Sucrose + water-alcohol extract	6	83	67	2.5
Sucrose + alcohol-ether extract	6	67	50	2.8
Sucrose + 100 mg choline chloride per kilogram	18	78	44	2.7
Sucrose + 200 mg choline chloride per kilogram <sup>1</sup>				
Sucrose + 500 mg choline chloride per kilogram <sup>1</sup>				
Starch	18	39	11	2.9
Extracted starch	12	17	8	3.3
Heated starch	6	50	33	3.1
Potato starch	6	Some ill but not due to hemorrhagic kidneys		0.8

<sup>1</sup> The animals of these groups were not entirely comparable with those of the other groups.

*Effects of succinylsulfathiazole (SST) on the protective action of starch*

At this point in the investigation, it was considered probable that the effect of the starch was due to an influence on the production by bacteria in the intestine of choline or some factor necessary for the utilization of choline stores or capable of affording protection in some other manner. To investigate this possibility, starch-containing diets with added SST were used.

The addition of 2% SST to the diet did not cause the expected increase in incidence of hemorrhagic kidneys (see table 4). Actually the death rate was somewhat less than without the SST. Again the food intake and growth were better than in the control animals. It was noted that the caeca of the animals receiving SST were, in every case, much larger, at the end of the experimental period, than those of the ani-

mals receiving starch or sucrose diets without SST. This, however, was probably due chiefly to dilatation, and was not altered by additions of choline.

In the 2 later experiments utilizing SST, biotin and para-amino benzoic acid were omitted from the diet. This did not alter the results.

TABLE 4  
*Effects of succinylsulfathiazole (SST).*

DIET	NUMBER OF ANIMALS	HEMORRHAGIC <sup>1</sup> KIDNEYS	DEAD WITH HEMORRHAGIC KIDNEYS	AVERAGE DAILY WT. GAIN FIRST 5 DAYS
		%	%	gm
Starch + 2% SST	14	43	7	2.4
Starch + 2% SST + 0.2% choline chloride	6	0	0	2.3
Starch	14	50	28	2.3
Sucrose	13	92	54	1.7

<sup>1</sup> The surviving animals were autopsied on the tenth day.

### *Choline content of the feces*

Significant amounts of choline were not found in the feces on any of the choline-deficient diets.

### *Effect of choline on the consistency of the diet mixtures*

The basal diet, particularly when made up with sucrose, was soft and sticky. When choline was added, the mixture immediately became much firmer and more granular. As little as 5-10 mg of choline chloride per 100 gm of diet produced a noticeable change in consistency. The mechanism of this effect was not extensively investigated but it seemed probable that it was due to a change in the physical state of the mixture not entirely explained by the hygroscopic effect of the choline chloride.

### *Pathological observations*

Most of the animals which became ill on the choline-deficient diets, exhibited the renal lesions previously described by

others, consisting of intense vascular congestion and some hemorrhages, and necrosis of the renal tubules particularly in the cortex. Some of the animals which were killed while still apparently healthy exhibited hyperemia and congestion of the vessels in the capsule and adjacent tissue.

In the acute stages of illness, the urine usually contained albumin, and sometimes appeared red but was not found to contain blood. In the animals which survived the acute stages, the resolution of the lesions was much like that described by Christensen ('42). However one of a small group of animals maintained on the choline-deficient diet for 4 months, died, apparently of renal insufficiency, 3 weeks after being placed again on the stock diet, and the kidneys were found to be severely scarred.

The incidence of intraocular hemorrhages was a little more than one-tenth that of hemorrhagic kidneys. In most cases the hemorrhages appeared to originate near the periphery of the lens. Ophthalmoscopic examination was attempted in a few animals where the hemorrhages were not so extensive as to make visualization impossible. No lesions to account for the hemorrhages were observed. Some animals which exhibited intraocular hemorrhages survived and were placed on the stock diet and observed for prolonged periods. In cases of massive hemorrhages, there was organization of the blood with fibrosis and loss of vision, while slight hemorrhages were resorbed with little visual impairment.

Most of the animals with hemorrhagic kidneys had livers which appeared slightly to markedly fatty. Two animals on the starch diet, which died presumably of hemorrhagic kidneys, were found on autopsy to have an acute focal necrosis of the liver, with grossly normal kidneys.

#### DISCUSSION

Fletcher, Best and Solandt ('35), using the method of acetylation and bioassay, reported a value for the choline content of corn starch higher than that found in the present study. It is possible that this discrepancy represented a real

difference in the choline content of the 2 starches. It is doubtful, however, that even the value reported by the investigators referred to, is sufficient to account for the anti-hemorrhagic-kidney effect of the starch. Furthermore, as has already been pointed out, the extracted starch, from which a significant portion of the choline had been removed, was just as effective as the unextracted starch.

Since others have experienced no difficulty in producing the hemorrhagic-kidney syndrome on diets containing 5 to 6% yeast, which must have contained at least 200 mg of choline per kg of diet according to the data of Engel ('42), it appeared unlikely from the beginning that the protective effect of starch was due entirely to choline. It was thought more probable that the starch contained other substances with labile methyl groups, not precipitated by reineckate in alkaline solution, or factors affording protection through some other mechanism, and that this study might provide an opportunity to isolate and identify the active substances. Engel ('42) observed that dried beef liver gave greater protection when fed with a choline-deficient diet than was accounted for on the basis of its choline content as determined chemically, and Lucas, Norris and Heuser ('46) reported that crude diets contained more "choline" by the microbiological method than by the reineckate determination. However, the inactivity of the starch-extracts, in spite of the fact that the extractable substances of the starch were not completely removed as judged by the choline content of the extracts, made the presence in starch of significant amounts of other protective factors unlikely. The diets containing the starch-extracts had a higher choline content, by a factor of approximately seven-fourths, and a much higher relative content of many of the other extractable substances, than the diets containing starch itself. The possibility remains, nevertheless, that the extracts may have contained some substances in lower proportion. More complete extraction of smaller amounts of starch, or of equal or greater amounts with elimination of the choline, would dispose of this uncertainty only if a positive result

were obtained in the form of greater protection by the extracts than accounted for on the basis of choline content, and this did not seem sufficiently likely to justify the laborious procedure.

The question of the effect of the starch on the intestinal synthesis of choline remained. du Vigneaud and associates ('39) demonstrated that labile methyl groups, which are an essential part of the choline molecule, cannot be synthesized in the body of most rats in necessary amounts, and constitute a dietary requirement, though an occasional rat may be able to grow on the methyl-free, homocystine-containing diet (with the carbohydrate in the form of sucrose). After feeding  $D_2O$  with a casein diet, it was shown (du Vigneaud, Simmonds, Chandler and Cohn, '45) that the methyl groups of choline isolated from the animals, contained a small amount of deuterium, indicating that some synthesis of methyl groups had occurred, probably in the intestine. Other investigators (Bennett, Medes and Toennies, '44) have observed more consistent growth on methyl-free diets, due, in Bennett's ('46) opinion, to a difference in the pre-experimental diet, and have obtained inhibition of growth by addition of succinylsulfathiazole to the diet (Bennett and Toennies, '46). However, since growth continued for a month after adding the SST, and was not resumed when the SST was removed without first returning the animals to the stock diet (Bennett, '46), it is not entirely clear what the effect of the SST was.

It has been shown that the bacterial flora of the intestine may be influenced by the composition of the diet (Herter and Kendall, '09), and that the amounts of vitamins produced by the intestinal bacteria may also be modified by changing the diet (Guerrant and Dutcher, '34). It seems, though, that if the protection afforded by starch against hemorrhagic kidneys was due to an increase in the synthesis of a protective factor in the intestine, that it would have been prevented by the SST. The results with SST did not rule out a decreased production of an aggravating factor as a result of substituting the starch for the sucrose, and it is possible that the choline-producing organisms were resistant to the SST in the con-

centration used, or that the 5- to 8-day period necessary for the development of the hemorrhagic syndrome was not long enough for the maximum effect of the SST.

Handler ('46) noted that hemorrhagic kidneys did not occur on diets with the carbohydrate in the form of lactose or galactose, apparently because of the general nutritional failure on these diets. This explanation was not applicable to the results obtained in this study with starch, for the nutritional state remained good.

The observation by others that the incidence of hemorrhagic kidneys in animals on choline-deficient diets is less when the animals are caged together than when kept in separate cages, suggested that choline determinations on the feces might provide evidence of intestinal synthesis. Biological assay of the feces for protective factors might have given more information than the chemical determinations.

The number of animals used was too small to permit definite conclusions regarding the apparent enhancement of the protective action of starch due to its extraction.

The acute focal necrosis of the liver and chronic renal injury of fatal degree, which were observed in a few animals in this study, have not been noted in similar studies by most investigators. Although the animals were apparently healthy in the pre-experimental periods, these lesions could not be attributed with certainty to choline deficiency. The intraocular hemorrhages bore at least a superficial resemblance to those of unknown etiology which occur fairly commonly in young men, and while it does not seem likely that the human disease is due to choline deficiency in most cases, the possibility that the defect may be related through other common factors to that in the experimental syndrome should be considered. The nature of the rather widespread vascular disturbance produced by choline deficiency, which may even be responsible for the renal necrosis, perhaps as a result of renal anoxia, has not been definitely determined or extensively investigated. The similarity of the renal lesions of the rats to those seen

in the human syndromes characterized by acute bilateral cortical necrosis, has been commented on by Christensen ('42).

#### SUMMARY

The incidence of illness and death due to the hemorrhagic-kidney syndrome in young rats on a choline-deficient diet, was significantly less when the diet contained corn starch, than when the carbohydrate was in the form of sucrose.

This effect of the starch was more than that produced by the addition to the sucrose diet, of more choline than was contained by the starch. Addition of increments of choline resulted in more rapid growth and a later appearance of hemorrhagic kidneys, before there was a significant decrease in the incidence of the syndrome.

Residues from extracts of the starch did not afford protection when added to the sucrose diet, and extracted starch was at least as effective as unextracted starch.

Addition of 2% succinylsulfathiazole to the starch-containing diet, did not diminish the protective action of the starch, and on none of the choline-free diets were significant amounts of choline found in the feces. The caeca of the animals receiving succinylsulfathiazole became markedly dilated.

In spite of the failure to obtain evidence of an increased bacterial synthesis of choline on the starch-containing diet, some effect within the intestinal tract appeared to be the most probable explanation of the protection afforded by starch, though the possibility of other protective factors in the starch was not entirely eliminated.

The lesions which were observed on the choline-deficient diets, and the significance of the vascular disturbance, were discussed.

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# THE QUALITY OF THE DIET AND THE CONSUMPTION OF SUCROSE SOLUTIONS<sup>1</sup>

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In 1939 the national expenditure for soft drinks was about \$400,000,000 (Reid, '43). The following studies of the past 4 years have attempted to use the white rat to determine some of the factors that influence the consumption of these beverages which consist chiefly of a 10% solution of sugar with flavors, coloring matter and acids such as phosphoric and citric. Previous studies have already been published in regard to the etching of the enamel of the teeth by these beverages (Bieri, McCay, Restarski and Gortner, '46).

## EXPERIMENTAL

### *The water consumption of the white rat*

Eleven rats were used to measure the water consumption when fed our stock diet.<sup>2</sup> Calibrated glass tubes were used for these measurements. Rats were about half grown at the start of the study. Six studies were made covering the months of August, September and October. The results are summarized in table 1.

<sup>1</sup>These experiments were started at the Naval Medical Research Institute, Bethesda, Maryland, and concluded in the Animal Nutrition Laboratory, Cornell University, Ithaca, New York. Assistance in the earlier studies was given by June Sullivan and R. A. Gortner, Jr. This research was financed in part by a grant from the Office of Naval Research to determine factors responsible for the deterioration of gums, teeth and vestment tissues.

<sup>2</sup>G.L.F. Dog Meal produced in Canandaigua, New York.

*The relative consumption of sucrose solution  
and water as a rat grows*

A series of exploratory studies using 140 rats was made to determine if the growing rat would tire of the daily ingestion of sucrose solutions, or sucrose-phosphoric acid as found in cola beverages. Paired rats were used to compare the relative amounts of water and sucrose solution consumed at different periods from weaning until the rats were 100 days old. In part of the trials, the rats were not used until they weighed 100 gm.

Detailed data from these studies are not presented since the results were the same in each trial. A period of 5-10 days was required for most rats to develop their taste for sucrose solutions, so that daily performance was consistent. After this the amount drunk increased regularly until the experiment terminated after 70 days.

In one typical study 28 rats were divided into 2 groups. One group was given sucrose solution and the other group water. The animals were caged individually. During the first 28 days the mean water consumption per day was 22 ml. Per 100 gm of body weight per day it was 19 ml. During the next 42 days the comparable values were 21 ml per day and 12 per 100 gm of live weight.

During this same period those given sucrose consumed during the first period a volume each day of 28 ml, or 24 ml per 100 gm of live weight. During the next 42 days the mean consumption was 46 ml per day and 22 per 100 gm of live weight.

The water consumption of a growing rat increases only slightly as it grows and declines in relation to its total body weight. The consumption of sugar solutions increases as the rat grows and maintains a fairly constant ratio to the live weight.

In the course of these exploratory studies, other variables were introduced for comparison such as a commercial cola beverage as sold, a water solution containing  $\text{H}_3\text{PO}_4$ , (18 mg per liter) and a 10% sucrose solution containing this same

amount of  $H_3PO_4$ . All solutions containing 10% sucrose gave the same results so neither the phosphoric acid nor other constituents of the commercial cola beverage, such as caffeine, modified the amount drunk by the rat.

*The effect of level of sugar in the diet upon the drinking of sugar solution*

In feeding men in the Navy, the proposal had been made that less soft drinks would be purchased if more sugar were employed in the ration. Two diets were devised to determine if sugar in the diet might modify the rat's consumption of sugar solution. The percentage composition of diet A was as follows: sucrose 48, salt mixture 4, casein 25, dry brewer's yeast 10 (mixed 1/50 with irradiated yeast), hydrogenated cooking fat 10, and cod liver oil 3. Corn starch was substituted for sucrose in diet B.

Young adult rats were divided into 2 groups and caged individually for this study. Each had access to water and 10% sucrose solution. In the course of 4 weeks, (table 2) no difference was found between those fed the diet rich in sugar contrasted with those fed the starch diet. Very little water was drunk. The mean consumption of sucrose solution was 60 and 61 ml per day.

Using the same animals a second trial was run feeding diets still richer in carbohydrates. In this case the sugar or starch was mixed to comprise 74% of each diet while the per cent of all other constituents was cut in half. This was done by mixing equal weights of sugar with diet A, and starch with diet B. In table 3 these new diets are designated  $A_1$  and  $B_1$ .

The results (table 3) provide no evidence that the sugar level of the diet modifies the amount of solution drunk by the rat. However, in the second experiment the quality of the diet was reduced in essential nutrients by dilution with carbohydrates. The amount of sucrose solution consumed by every individual on both the starch and sugar diets was reduced

TABLE 2

*Rat experiment using high sugar and starch diets, and 10% sugar solution and water (7-12-11 to 8-8-11).*

RAT NO	FOOD CONSUMED		SUGAR SOLUTION CONSUMED		WATER CONSUMED		BODY WEIGHT	
	Total	Per day	Total	Per day	Total	Per day	Gained 7/12-8/8	On 8/8
	gm	gm	ml	ml	ml	ml	gm	gm
Diet A (Sugar 48%)								
234	274.4	10	1956	70	56	2	63	106
235	314.7	12	1122	40	60	2	56	435
236	267.7	10	1926	69	31	1	66	439
237	266.4	10	1614	59	71	3	73	333
238	263.8	9	1816	65	62	2	83	411
239	233.4	8	1617	58	57	2	32	332
Aver.	275.1	10	1680	60	56	2	62	393
Diet B (Starch 48%)								
240	315.5	11	1249	45	110	4	54	362
241	208.0	7	1920	69	79	3	30	372
242	264.2	9	2104	73	45	2	47	422
243	172.1	6	1795	64	15	1	..	364
244	207.1	7	1495	53	37	1	37	325
Aver.	233.4	8	1713	61	57	2	34	369

TABLE 3

*Rat experiment using high sugar and starch diets (8-9-11 to 9-6-11).*

RAT NO	FOOD CONSUMED		SUGAR SOLUTION CONSUMED		VOLUME OF WATER CONSUMED		BODY WEIGHT	
	In 28 days	Per day	Total	Per day	Total	Per day	Gained	Final
	gm	gm	ml	ml	ml	ml	gm	gm
Diet A <sub>1</sub> (Sugar 74%)								
234	336	12	1405	50	75	3	19	425
235	438	16	764	27	64	2	21	456
236	355	13	1264	45	56	2	13	452
237	295	11	894	32	39	1	1	334
238	257	9	1627	58	102	4	1	412
239	224	8	1232	44	65	2	— 23	309
Aver.	318	11	1198	43	67	2	5	
Diet B <sub>1</sub> (Starch 74%)								
240	449	16	677	24	101	4	30	392
241	275	10	1516	54	91	3	7	379
242	278	10	1802	64	74	3	11	433
243	291	10	1152	41	47	2	— 2	362
244	231	8	1250	45	60	2	— 3	322
Aver.	305	11	1279	46	75	3	9	

during this period of 4 weeks. This was the first of a number of observations that suggested the possibility of using the volume of sugar consumed by the rat as an indicator of deficiencies in diets.

### *Calcium in the diet and sucrose consumption*

The experience with diets rich in carbohydrates indicated that the composition of the diet influenced the rat in the amount of sucrose beverage consumed, but did not indicate which of many variables might be responsible.

Additional clues were found in the course of another exploratory experiment. Plans were made to determine whether the teeth of growing rats were more subject to the etching effect of cola beverages when these rats were fed diets very low in calcium. A food mixture was prepared with the following percentage composition: corn meal 76, cottonseed oil 10, corn germ 5, dried brewer's yeast 5, cod liver oil 3, and sodium chloride 1. A second diet was prepared that would provide the animal with less calcium by mixing each 100 gm of the above feed with 50 mg of sodium oxalate.

Sixteen young rats were divided into 2 groups and placed upon the above diets. Each of these was subdivided so that 6 were given water to drink, 5 cola beverage and 5 of them 10% sucrose solution.

The original purpose of the experiment had to be abandoned because the consumption of the cola beverage and sugar solutions was so low that comparison could not be made with preceding studies. The average was 9 ml of sugar solution in contrast to 12 ml of water daily per 100 gm of live weight. In spite of the low calcium diets these rats all lived and doubled their body weights in 12 weeks.

In contrast a similar experiment was made with young hamsters during the same period and on the same diets. All of the hamsters died, indicating a much greater sensitivity of this species to low calcium diets.

At the end of 12 weeks, individuals were given supplements of bone meal or calcium carbonate or casein. There was some



response in each case as indicated by the drinking of more sucrose solution but this was the most marked in the case of those fed bone meal. Rats kept on the low calcium diet but changed from sugar solution to water responded at once by an increase in fluid consumption.

These exploratory studies indicated further that the amount of 10% sugar solution drunk by a rat represented a response to the quality of the diet. One of the factors indicated was calcium.

### *Response to calcium, vitamins and casein*

Two years after the above observations were made at Bethesda, Maryland, this study was extended. External conditions were more uniform since the rats were now kept in an air-conditioned laboratory.

Two experiments were completed following the earlier method of diluting an adequate basal diet with starch. In this case the basal diet consisted of the stock feed used in rearing our breeding colony. In a preliminary trial with growing rats 3 parts of this basal diet were mixed with 1 of corn starch. In paired feeding trials young rats grew as well upon this diluted diet as upon the stock diet itself. Furthermore, the consumption of 10% sugar solutions by rats upon both diets was the same, indicating that rats sensed no deficiency in the basal diet.

Earlier experience had indicated this stock diet could be diluted even further without any reflection in the development of growing rats provided the diet were mixed with 3 parts of corn starch. Thus the protein was reduced to about 7%.

This mixture was used in 2 studies, 1 with adult rats 5 months of age, and 1 with rats 5 weeks old. In each case rats were paired on the basis of sex and litter. One rat of each pair was fed the stock diet: the other was fed the diet diluted with 3 parts of starch. Rats were caged individually and allowed all of the 10% sucrose solution they desired. The amount was measured daily using 100-ml graduated tubes.

In each study before adding supplements for test, a preliminary period of 8 weeks for the adult rats and 4 for the young ones was allowed until the differences between the consumption of sugar solutions by the 2 groups became significant.

After this, various supplements were added first singly, then in pairs and finally in trios. These additions included water-soluble vitamins, calcium gluconate, yeast, vitamin A, and casein.

The response could be judged by comparing the volume of solution drunk by the test animals and the controls. These supplements are indicated in tables 4 and 5.

The response of the older group to calcium in 3 different periods is evident (table 4). The consumption of the sugar solution in each case actually exceeded the control. In light of the recent findings (Kane and McCay, '47) that a higher level of calcium is required to maintain a rat in calcium balance as it grows older, this result may be partly explained. None of the other substances tested gave a response like calcium.

The results with the growing rats (table 5) were similar inasmuch as they responded to calcium but not when this was the sole addition. Neither calcium alone nor in combination with a water-soluble vitamin as riboflavin or a source of protein such as casein gave a response. However, when the supplement consisted of calcium, vitamin A and yeast or casein, the consumption of the sucrose solution exceeded the control.

Thus the growing rat would seem to sense a combined deficiency of calcium, vitamin A and protein but not one with respect to the water-soluble vitamins contained in the yeast. Inasmuch as this study was not made with synthetic diets known to be deficient in water-soluble vitamins, the only definite conclusion is that under these conditions the volume of sugar solution consumed may serve as a criterion of calcium deficiency and possibly of others such as protein.

Recent exploratory studies indicate that old rats in the last third of life may lose their abilities to sense some common deficiencies that are readily sensed in the first third of life.

TABLE 4  
Consumption of sugar solution by young adult rats.

SUPPLEMENT PER DAY	TIME IN WEEKS	PAIRS NO. OF	AVERAGE ML DRUNK PER RAT BY PERIODS <sup>1</sup>		
			Experimental group	Control group	Signifi- cance
Preliminary period					
			$\frac{1}{2}$ basal plus $\frac{1}{2}$ starch	Basal	<sup>2</sup>
None	9	10	$64.1 \pm 19.0$	$59.1 \pm 24.4$	
			$\frac{1}{2}$ basal plus $\frac{1}{2}$ starch	Basal	<sup>2</sup>
None	8	9	$48.4 \pm 19.7$	$66.9 \pm 20.5$	
First supplementary period					
50 $\mu$ g <sup>1</sup> thiamine	3	3	$54.2 \pm 18.4$	$84.8 \pm 25.4$	<sup>2</sup>
200 $\mu$ g riboflavin	3	2	$33.7 \pm 7.2$	$67.4 \pm 12.5$	<sup>2</sup>
100 mg calcium	3	2	$69.8 \pm 28.3$	$69.0 \pm 21.0$	0
2.5 gm yeast	3	2	$38.1 \pm 10.8$	$63.5 \pm 13.5$	<sup>2</sup>
Second supplementary period					
1.7 gm casein	5	3	$54.2 \pm 28.6$	$81.0 \pm 30.6$	<sup>2</sup>
1.7 gm casein + 6 $\mu$ g vitamin A	5	3	$58.7 \pm 26.5$	$83.4 \pm 9.5$	<sup>2</sup>
1.7 gm casein + 6 $\mu$ g vitamin A + 100 mg calcium					
	5	3	$75.4 \pm 29.2$	$60.7 \pm 14.6$	<sup>2</sup>
Third supplementary period					
1.7 gm yeast	5	3	$49.9 \pm 16.9$	$80.3 \pm 33.5$	<sup>2</sup>
1.7 gm yeast + 6 $\mu$ g vitamin A	5	3	$67.9 \pm 16.4$	$77.7 \pm 7.8$	<sup>2</sup>
1.7 gm yeast + 6 $\mu$ g vitamin A + 100 mg calcium					
	5	3	$94.1 \pm 16.9$	$62.3 \pm 8.8$	<sup>2</sup>

<sup>1</sup> Means  $\pm$  standard deviations.

<sup>2</sup> Significant at 1% level.

TABLE 1  
Consumption of sugar solution by young adult rats.

SUPPLEMENT PER DAY	TIME IN WEEKS	PAIRS NO. OF	AVERAGE ML DRUNK PER RAT BY PERIODS <sup>1</sup>		S.E.
			Experimental group	Control group	
Preliminary period					
			$\frac{1}{3}$ basal plus $\frac{1}{3}$ starch	Basal	
None	9	10	61.1 $\pm$ 19.0	59.1 $\pm$ 21.4	
			$\frac{1}{3}$ basal plus $\frac{1}{3}$ starch	Basal	
None	8	9	48.1 $\pm$ 19.7	66.9 $\pm$ 20.5	
First supplementary period					
50 $\mu$ g <sup>1</sup> thiamine	3	3	54.2 $\pm$ 18.4	84.8 $\pm$ 25.4	2
200 $\mu$ g riboflavin	3	2	33.7 $\pm$ 7.2	67.4 $\pm$ 12.5	2
100 mg calcium	3	2	69.8 $\pm$ 28.3	69.0 $\pm$ 21.0	0
2.5 gm yeast	3	2	38.1 $\pm$ 10.8	63.5 $\pm$ 13.5	2
Second supplementary period					
1.7 gm casein	5	3	54.2 $\pm$ 28.6	81.0 $\pm$ 30.6	2
1.7 gm casein + 6 $\mu$ g vitamin A	5	3	58.7 $\pm$ 26.5	83.4 $\pm$ 9.5	2
1.7 gm casein + 6 $\mu$ g vitamin A + 100 mg calcium					
	5	3	75.4 $\pm$ 29.2	60.7 $\pm$ 14.6	2
Third supplementary period					
1.7 gm yeast	5	3	49.9 $\pm$ 16.9	80.3 $\pm$ 33.5	2
1.7 gm yeast + 6 $\mu$ g vitamin A	5	3	67.9 $\pm$ 16.4	77.7 $\pm$ 7.8	2
1.7 gm yeast + 6 $\mu$ g vitamin A + 100 mg calcium					
	5	3	94.1 $\pm$ 16.9	62.3 $\pm$ 8.8	2

<sup>1</sup> Means  $\pm$  standard deviations.

<sup>2</sup> Significant at 1% level.

# PROTEIN ASSAY BY RAT GROWTH:

A COMPARISON OF (A) LITTER MATES VS. RANDOMLY SELECTED MALES,  
AND (B) MODERATE RESTRICTION OF FOOD INTAKE VS.  
AD LIBITUM FEEDING

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ONE FIGURE

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Numerous laboratories have adopted rat growth methods as means for an evaluation of proteins and protein hydrolysates. Direct comparison of results among several laboratories shows striking qualitative agreement; quantitative variations are encountered as direct sequelae of a host of variables. To name only a few these would include ration composition, animal strain, level of test feeding and caloric intake of animals.

Studies of the roles of some of these variables under homogeneous conditions have been reported from many laboratories. Mitchell ('44) and Barnes et al. ('45) have recently compared *ad libitum* and paired feeding technics. Barnes and Bosshardt ('46) have demonstrated the effects of level of protein fed on rat growth and protein efficiency. Forbes et al. ('46) showed the consequences of variation of fat level in the ration on rat growth. Vitamin supplement effects have been explored by Bosshardt et al. ('45), Harte and Travers ('47), Harris and Hove ('47) and others. Whether streptogenin plays a role in protein utilization has been explored by Woolley ('45) and Womack and Rose ('46). Comprehensive bibliographies are given by Block and Mitchell ('46-'47) and Mitchell and Hamilton ('29).

## SUMMARY

Rats fed an adequate stock diet drink about 12 ml of water or 24 ml of 10% sucrose solution per day per 100 gm of live weight. The water consumption of growing rats in relation to body weight declines progressively as the rats mature, while the sugar solution drunk is relatively constant.

No evidence has been found that rats tire of a sugar solution. Diets rich in sugar or starch produce no decrease in the drinking of sucrose solutions unless the high carbohydrate dilutes essential nutrients to a low level.

When fed diets low in calcium rats refuse to consume cola beverage or sucrose solution except in amounts to provide water. *Under such conditions the amount of sucrose solution drunk is lower than normal water consumption.*

When an adequate stock diet was diluted with 3 parts of corn starch, the amount of sucrose solution consumed was observed to depend upon supplementing the diet with calcium in the case of adult rats; in the case of growing rats, a high level of sucrose solution was drunk only when the diet was supplemented with protein, vitamin A and calcium.

An adult rat drinking daily 72 ml of sucrose solution excreted 43 ml of urine in contrast to a rat drinking 40 ml of water and excreting 15 ml of urine.

Rats lactating heavily drank 69-71 ml of sucrose solution and 17-24 ml of water.

Exploratory studies indicate that the rat's ability to detect certain dietary deficiencies may decrease with advancing age.

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The surviving 49 randomly selected males were divided into 4 groups of 10, and 1 of 9, adjusted only to give corresponding average weights. These groups were designated A', B', C', D', and E' and the 10 groups were maintained on the casein ration according to the schedule, groups A, B, C and A', B', C'

TABLE 1  
*Composition of ration used.*

Lard . . . . .	90.0 gm	Thiamine . . . . .	5 mg
Salts <sup>1</sup> . . . . .	40.0 gm	Riboflavin . . . . .	8 mg
Cod liver oil . . . . .	20.0 gm	Niacin . . . . .	13 mg
Wheat germ oil . . . . .	10.0 gm	Calcium pantothenate . . . . .	13 mg
Choline . . . . .	1.8 gm	Pyridoxine . . . . .	6 mg
Casein <sup>2</sup> to yield 1.6% nitrogen			
Starch q.s. to make a total of 1 kg			

<sup>1</sup> GBI (General Biochemicals, Inc.) salt mixture was used.

<sup>2</sup> Labco Vitamin Free casein (Borden) was used.

TABLE 2  
*Distribution of groups.*

LITTER MATES			RANDOMLY SELECTED MALES	
Group	Distribution by sex		Group	Average body weight
				gm
A	4 ♂	6 ♀	A'	78.3 ± 2.2
B	4 ♂	6 ♀	B'	78.5 ± 3.4
C	4 ♂	6 ♀	C'	77.9 ± 2.7
D	6 ♂	4 ♀	D'	72.1 ± 3.1
E	6 ♂	4 ♀	E'	74.0 ± 2.4
				gm

<sup>1</sup> Indicated limits represent standard error (s.e.) of the mean, where

$$\text{s.e.} = \sqrt{\frac{(\sum (X - \bar{X})^2)}{n(n-1)}}; \text{X being}$$

individual values and  $\bar{X}$  the group mean, n representing the number of individuals. This convention is employed in all following tables, where relevant.

being restricted to not more than 10 gm of ration daily; groups D, E, and D', E' were fed *ad libitum*. Water consumption was not restricted for either group. Food intakes were measured and spillage was collected daily. The animals were weighed weekly.

In the establishment of a standard method for evaluating a protein or protein hydrolyzate for pharmacopoeial purposes, for example, certain requirements must be met. The technic decided upon must be relatively simple, yet reproducible; it should give uniform results in the hands of any laboratory group engaged in such work and must have good discriminatory capacity. For both control and enforcement purposes the test devised must not permit making too many errors of either the first or second kind; namely, rejection of satisfactory preparations or acceptance of sub-standard preparations.

This report deals with a survey of 2 characteristics of experimental design which might be expected to play a role in the discriminatory capacity of such a test, namely, (1) the use of litter mates and (2) the restriction of dietary intake imposed by putting an upper limit on the amount of food offered. Data on roles played by these factors do not seem to be available in the literature. The imposition of an upper limit on amount of food offered has been successfully used in protein regeneration studies conducted on the rat by Frazier et al. ('47). In the study reported in the present paper a total of 99 animals were observed when fed an identical casein ration and the data subjected to statistical analysis.

#### EXPERIMENTAL

Table 1 shows the ration used in these experiments. This diet is estimated to provide 4.5 cal. per gm. Ten litters (6 rats each) and 50 randomly picked males<sup>1</sup> were caged separately in an air-conditioned animal room (76°F.) and put on our basic casein ration. After 1 week 2 animals had died and 1 had failed to gain more than 5 gm and was therefore discarded.

At this time the remaining litter mates were divided into 5 groups of 10 to which 3 and 2 represented matched units with respect to litter, sex and average weight (see table 2).

<sup>1</sup> From Rockland Farms. The cooperation of Harry G. Herrlein of Rockland Farms in making these litters and the randomly selected males available is greatly appreciated.



occurrence of much greater standard errors. Protein efficiencies were not very different for both random males groups, but the values for the litter mates groups do not agree well.

When the weight data for the sibling of the rat in group E which died were discarded from group D and an analysis of variance carried out, the difference between litter mates is, surprisingly, statistically significant, with an associated probability of about 0.03. Similar examination of the corresponding protein efficiency data also reveals a significant statistical difference, with an associated probability of about 0.01.

TABLE 4

*Weight gains and protein efficiencies of groups fed ad libitum.*

GROUP	n	WEIGHT GAIN	PROTEIN EFFICIENCY	REMARKS
		gm		
D	10	69.7 $\pm$ 4.3	2.18 $\pm$ 0.08	Litter mates
E	9	82.3 $\pm$ 4.7	2.45 $\pm$ 0.06	Groups
D'	10	81.6 $\pm$ 7.4	2.44 $\pm$ 0.10	Groups of
E'	9	75.1 $\pm$ 2.3	2.36 $\pm$ 0.06	randomly selected males

Of some interest, too, is the fact that the spillage average for the 58 rats whose rations were restricted was only 8.8 gm during the 28-day period, while the 38 animals fed *ad libitum* apparently lacked the incentive of restriction to acquire neater eating habits and showed an average spillage of 39.5 gm.

At the end of 6 weeks on these rations the data were again analyzed; the relative results were entirely unchanged and therefore are not reported in detail.

#### DISCUSSION

Since the basic experimental design was such as to exclude the existence of any differences between rations the statistical conclusions apply to situations apt to be encountered more-or-less rarely in assays where no true differences exist between rations being compared. We feel that these data suggest that the added refinement of the litter mate technic offers

## RESULTS

During the first week 2 rats died. The deaths occurred in the litter groups C and E and were of animals that had gained 9 and 16 gm, respectively, during the pre-conditioning period. During this first week, too, 3 animals gained less than 5 gm, although they had respectively gained 8, 22 and 25 gm previously. One additional fatality occurred in group B' during the third week of the test period.

At the end of 4 weeks the weight gains were scored and analyzed. The results will be considered, first, for those animals restricted to 10 gm of ration daily. Table 3 gives the weight gains, and protein efficiencies, together with their

TABLE 3

*Weight gains and protein efficiencies of groups with restricted food intake.*

GROUP	n	WEIGHT GAIN	PROTEIN EFFICIENCY	REMARKS
		gm		
A	10	60.9 $\pm$ 2.0	2.39 $\pm$ 0.08	Litter mates Groups
B	10	62.2 $\pm$ 1.4	2.35 $\pm$ 0.05	
C	9	63.3 $\pm$ 1.7	2.37 $\pm$ 0.06	
A'	10	60.0 $\pm$ 2.8	2.42 $\pm$ 0.08	Groups of randomly selected males
B'	9	62.1 $\pm$ 1.8	2.40 $\pm$ 0.06	
C'	10	62.9 $\pm$ 2.1	2.38 $\pm$ 0.07	

standard errors. From this table it is evident that there is no significant difference between any 2 of the 6 groups, whence there appears to be no advantage in using litter mates as compared to randomly chosen males.

Since 1 of the litter mates in group C had died during the first week, the data for its siblings were segregated from the other 2 groups and an analysis of variance carried out by standard methods as described by Fisher ('41) and Snedecor ('40). This analysis showed no reduction in mean square value among litter mates.

The data coming from the groups fed *ad libitum* (table 4) showed strikingly different results. The mean gains were somewhat greater and more irregular, with the concomitant

diminish by almost 20% the food consumption from the *ad libitum* level.

It is most important to reduce the experimental variance as much as possible to help avoid making the errors, first, of reporting differences where none exist, and second, of failing to recognize differences which do exist. For example, previous experiments have suggested that randomly chosen males fed 10% casein *ad libitum* in somewhat similar rations gain on the average 80 gm in a 30-day period, with a variance, for a single animal, of  $400 \overline{\text{gm}}^2$  (The pooled variance for groups D' and E' is  $316 \overline{\text{gm}}^2$ ; the pooled mean, 78.5 gm.) When these were assumed to be the true parameters of the performance of casein it was possible to calculate for all experimental preparations whose true mean gain values ranged from 50 to 100 gm with constant variance ( $400 \overline{\text{gm}}^2$ ) the probability of making false decisions on a 90% criterion; i.e., preparations whose performance is poorer than 90% of the performance of casein are to be rejected.

When these estimations were made it appeared that a false decision would be made on the average 3 times in every 15 assays of products whose true values were in the range from 60–120% of a standard; further, 2 of these false decisions would involve acceptance of preparations poorer than 90% of casein while 1 would involve rejection of a preparation better than 90% of casein.<sup>2</sup>

With temporary acceptance of the parameters established by this study for animals fed casein on restricted intake (mean gain = 62 gm, variance =  $40 \overline{\text{gm}}^2$ ), the situation with respect to the probability of false decisions is substantially improved. Under these circumstances the likelihood of false decision is reduced to 1 in about 16 assays (with respect to the same range of products), and of every 12 false decisions 5 will be false rejections and 7 false acceptances, the 90% criterion being retained. The calculations are summarized in figure 1.

<sup>2</sup> Dr. William J. Youden has been kind enough to review our calculations and confirm these statements. His friendly interest is gratefully acknowledged.

no advantage under conditions where randomly grouped males from single shipments of not less than 50 animals from a reasonably homogeneous source of supply are used.

The sole advantage which might be expected to accrue from the use of litter mates when differing rations are undergoing comparison is greater homogeneity of the variance in groups so selected. Examination of the data in table 3 makes the application of the appropriate statistical test (Snedecor, '40) superfluous. It is obvious that the variances for groups A, B, and C and for groups A', B' and C' are equal and equally homogeneous.

On the other hand, the data in table 4 suggest that the variances associated with the growth responses in the 2 groups of randomly selected males may not be homogeneous; application of Bartlett's test gives a value for chi squared which just borders on conventional statistical significance.

In experiments such as those reported here one might postulate that if siblings of the same sex have greater similarity of growth potential than randomly chosen animals, then the variance within litters ought to be less than the variance between litters. Not only is this expectation not realized, but with the *ad libitum* fed animals the contrary result definitely emerges. Within the framework of these data the postulate is not supported and doubt is cast on the premise from which it arises. Of course these conclusions rest on data yielded by only 45 animals and definitive affirmation of the thesis must await experimentation on a larger scale.

The pitfalls in experiments of this sort are well exemplified by the results obtained with groups D and E. Unless one had *a priori* reasons to expect no difference, such a result would pass unchallenged in a routine test and the ration fed the animals in group D would be adjudged inferior whether the data were merely compared in a rough fashion or subjected to scrupulous statistical analysis.

There seems, however, to be little question that material improvement in precision results from the moderate degree of food intake restriction imposed in part of this experiment. For the whole experiment, the effect of restriction was to

response (and variance) of precisely the same order of magnitude as groups from the same stock which are paired with respect to sex and litter.

2. Preconditioning of the experimental population by feeding for 1 week on a 10% casein ration offers some improvement but does not entirely eliminate fatalities or irregular performance during subsequent weeks on test.

3. Partial restriction of food intake (to not more than 10 gm daily) reduces the mean growth response over 28 days on 10% casein rations by approximately 20% from the mean growth of *ad libitum* fed animals.

4. The variance for restricted fed animals is only about one-eighth to one-tenth that observed for *ad libitum* fed animals.

5. The reduction of variance offers approximately 3.5-fold improvement in the discriminatory capacity of an assay test set-up on a 90% criterion.

6. The physiological implications of the partial food restriction technic remain to be elucidated.

#### ACKNOWLEDGMENTS

The assistance of Dorothy Nicholson and Louis Quesal in this work is greatly appreciated.

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The benefits thus accruing for assay purposes are substantial and may outweigh the physiological objections that moderate dietary restriction might justify.

However, these are purely mathematical considerations, pessimistic to the extent that they do not take into account the correlation which exists between the growth responses of standard and test material subjected to parallel replication.

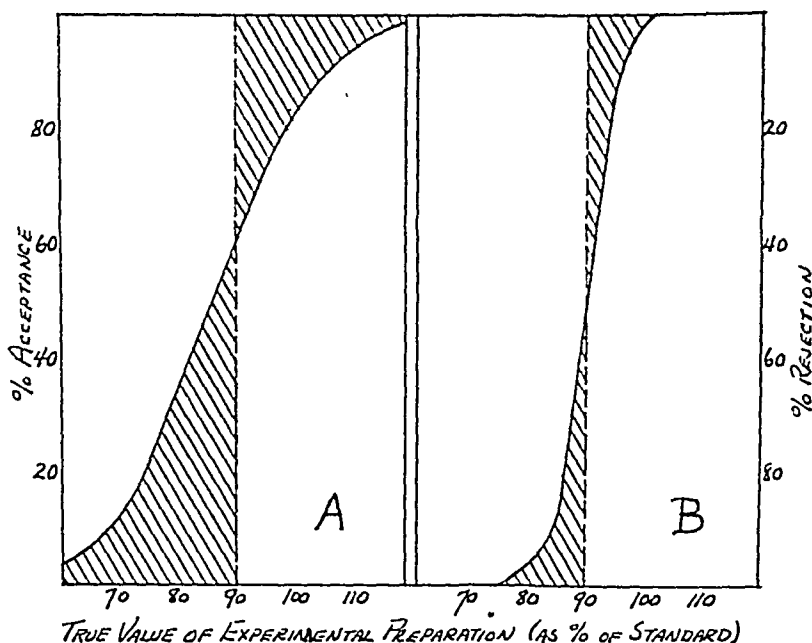


Fig. 1 Probability of acceptance and rejection of experimental preparations referred to a 90% of standard criterion. A — *ad libitum* fed animals ( $n = 10$ ; assumed parameters —  $M = 80$  gm,  $\sigma^2 = 400$   $\overline{\text{gm}}^2$ ). B — Animals fed with moderate restriction ( $n = 10$ ; assumed parameters —  $M = 62$  gm,  $\sigma^2 = 40$   $\overline{\text{gm}}^2$ ).

Cross hatched areas represent regions of false decision.

Before a final decision can be reached it will be necessary to explore more fully the physiological aspects of the restrictive technic. Experiments with this in view are in progress.

#### SUMMARY

1. Groups of 9 or 10 randomly chosen male albino weanling rats, selected from groups of 50 or more, give mean growth

# THE COMPARATIVE BIOLOGICAL AVAILABILITIES OF FERROUS SULFATE IRON AND FERRIC ORTHOPHOSPHATE IRON IN ENRICHED BREAD<sup>1</sup>

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THREE FIGURES

(Received for publication May 17, 1947)

According to the flour and bread enrichment program, as developed by federal and state agencies, the required iron should be added to the flour only in forms which are harmless and assimilable (Fed. Reg., '41). Primary consideration has naturally been given to the selection of iron preparations which exert no deleterious action upon the flour or bread. This technical requirement has somewhat obscured the basic nutritional motive for enrichment in that the degree of biological availability of the added iron has not been thoroughly considered. As has been pointed out by the Council on Foods and Nutrition of the American Medical Association ('41), obviously there is no nutritional advantage in adding an iron compound if this iron is not satisfactorily available to the body. Tobey and Cathcart ('41) pointed out that little information had been reported on the assimilability of iron compounds that were already in use for enrichment of flour.

Several forms of iron have been of practical interest for enrichment. The initial report on ferric phytate gave a somewhat favorable impression (Andrews, Evans and Huber, '41),

<sup>1</sup> Presented in part before the American Institute of Nutrition, Chicago, May 21, 1947 (Blumberg, H., and A. Arnold, '47, Fed. Proc., 6: 402).

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In view of the large extent to which enrichment of the nation's bread is conducted at the bakery, it was considered desirable to obtain a better quantitative comparison of the relative biological availabilities of ferrous sulfate and ferric orthophosphate by testing each form of iron at several levels. The compounds were fed in the form of enriched breads containing the different iron sources, so that the iron would be tested in the same form as used for human consumption. A secondary comparison with ferric chloride was also made because some investigators have used the latter compound rather than ferrous sulfate as a standard of high biological availability.

#### EXPERIMENTAL METHODS

##### *Diets*

The diet employed in these experiments was similar to that previously used (Blumberg and Arnold, '47); the composition is given in table 1. Casein was included in the diet as a supplement to the inadequate protein of the bread, so as to provide sufficient protein for optimal hemoglobin regeneration. Low-iron casein, containing approximately 15  $\mu\text{g}$  of iron per gm was prepared in the laboratory from skimmed milk. The low-iron salt mixture was prepared by modifying U.S.P. XI Salt Mixture no. 2 in the following manner. The ferric citrate was omitted, of course. Since the bread supplied sodium chloride, this also was omitted from the mixture and the amount of salt mixture used was reduced from the usual 4% of the diet to 3%. Furthermore, potassium biphosphate was substituted for sodium phosphate.

Four lots of bread were baked from the same lot of flour with special enrichment mixes that supplied the usual amount of thiamine, riboflavin, and niacin, but varied with respect to iron. One lot of bread was for the negative control and contained no added iron. The other 3 lots were enriched to provide approximately the following quantities of iron: (1) 13  $\mu\text{g}/\text{gm}$ , as ferric orthophosphate; (2) 42  $\mu\text{g}/\text{gm}$ , as ferrous sulfate; and (3) 21  $\mu\text{g}/\text{gm}$ , as ferric chloride. The ferric orthophosphate was from a batch actually used for commercial

enrichment; the exsiccated ferrous sulfate was U.S.P. grade, and the ferric chloride was C.P. grade.

The breads were air-dried at 37°C. to a moisture content of approximately 4% and were then ground for use in the diets. Iron analyses of the breads were made by a thiocyanate procedure (Eckert and Auerbach, '44). The iron content of the negative control bread was found to be about 12  $\mu\text{g/gm}$ . The actual analyses indicated that the supplemented breads had slightly more than the intended additional iron contents, as follows: ferric orthophosphate 132  $\mu\text{g/gm}$ , ferrous sulfate

TABLE 1  
*Composition of diet.*

BASAL MIXTURE		SUPPLEMENTS PER 100 GM BASAL MIXTURE <sup>1</sup>	
	%		mg
Bread (dried)	82	Thiamine hydrochloride ..	1
Casein (low-iron)	12	Riboflavin . . . .	2
Salt mixture (low-iron)	3	Pyridoxine hydrochloride	1
Corn oil .	3	Calcium pantothenate	4
		Niacinamide (nicotinamide)	2
		Choline chloride	100
		Inositol	100
		Copper (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ )	3
		Manganese (as $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ )	1.5

<sup>1</sup> Each rat received by stomach tube a weekly fat-soluble vitamin supplement equivalent to 2000 U.S.P. units of vitamin A, 400 U.S.P. units of calciferol (vitamin  $\text{D}_3$ ), and 10 mg of alpha-tocopherol.

43  $\mu\text{g/gm}$ , and ferric chloride 26  $\mu\text{g/gm}$ . By suitable dilution with the negative control bread, the iron-enriched breads were made into the series of test bread mixtures shown in table 2. The 4 levels of ferric orthophosphate iron increased in geometric progression by multiples of 2.5, as follows: 8.4, 21.0, 52.5, and 131.2  $\mu\text{g/gm}$  of bread, or 6.9, 17.2, 43.1, and 107.6  $\mu\text{g/gm}$  of diet. The 4 levels of ferrous sulfate iron increased by multiples of 2, as follows: 5.25, 10.5, 21.0, and 42.0  $\mu\text{g/gm}$  of bread, or 4.3, 8.6, 17.2, and 34.4  $\mu\text{g/gm}$  of diet. The 2 levels of added ferric chloride iron were made the same as the intermediate levels of ferrous sulfate, i.e., 10.5 and 21.0  $\mu\text{g/gm}$  of

bread or 8.6 and 17.2  $\mu\text{g/gm}$  of diet, in order to permit comparison of these 2 highly available iron preparations.

The iron contents of the various diets are given in table 2. The extraneous, non-bread iron in the diets was only about 2  $\mu\text{g/gm}$ . Diet 12 was prepared by addition of ferrous sulfate to the negative control diet 1 at a level of 244  $\mu\text{g}$  of iron per gm of bread, or 200  $\mu\text{g/gm}$  of diet. This provided a positive control to demonstrate the maximum rate of hemoglobin regeneration permitted by the basal diet in the presence of an amount of available iron known to be well within the optimal range.

TABLE 2  
*Iron contents of breads and diets.*

GROUP	BREAD		NO. RATS	IRON CONTENT OF BREAD	IRON CONTENT OF DIET
	Compound added	Iron added			
		$\mu\text{g/gm}$		$\mu\text{g/gm}$	$\mu\text{g/gm}$
1	None (negative control)		9	12.0	11.9
2	Ferric orthophosphate	8.4	8	20.4	18.8
3	Ferric orthophosphate	21.0	8	33.0	29.2
4	Ferric orthophosphate	52.5	9	64.5	54.9
5	Ferric orthophosphate	131.2	10	143.0	118.0
6	Ferrous sulfate	5.25	9	17.2	16.2
7	Ferrous sulfate	10.5	9	22.5	20.5
8	Ferrous sulfate	21.0	10	33.0	29.2
9	Ferrous sulfate	42.0	9	54.0	45.0
10	Ferric chloride	10.5	9	22.5	20.5
11	Ferric chloride	21.0	10	33.0	29.2
12	Ferrous sulfate (positive control)	244.0	7	256.0	212.0

### *Animal experimentation*

Albino rats of the Sherman strain were prepared for iron-deficiency studies by special feeding precautions generally similar to those described by Elvehjem and Kemmerer ('31). At about 25 days of age the weanling rats were removed to individual galvanized cages in which there was no exposed iron or rust. Anemia was induced by feeding certified cow's milk supplemented with cupric sulfate and manganous sulfate equivalent to 1.8 mg of copper and 1.4 mg of manganese

per liter. After the rats had been on the iron-depletion diet for 35 days, hemoglobin determinations were made on tail blood by the alkaline hematin method, as adapted for the Klett-Summerson colorimeter. Except for 12 somewhat resistant animals, the rats were found to be sufficiently anemic for test purposes, i.e., the hemoglobin values were 2.5-5 gm/100 ml, with an average of about 3.9 gm/100 ml.

The animals were divided into groups of 10 rats each, except for the positive control group 12, which had only 7 rats. Weight and sex distributions were similar in the various groups. Occasional mortality during the experiment reduced the numbers slightly, so that the final test groups contained 8-10 rats each, as shown in table 2. The experimental diets were then fed for 4 weeks, hemoglobin determinations and weighings being made at the end of each week. Groups 1, 2, 3, and 6, which were regenerating hemoglobin at slow rates, were continued on experiment for as long as 9 weeks for determination of the length of time required to reach a hemoglobin value of 10 gm/100 ml, i.e., close to the beginning of the normal range.

## RESULTS

### *Ferrous sulfate and ferric orthophosphate*

The general nature of the results of the comparison between ferrous sulfate and ferric orthophosphate is shown by the hemoglobin curves in figure 1. The rats fed the negative control diet and the 2 lower levels of ferric orthophosphate had not shown any increase in their hemoglobin concentration values by the end of the first week. However, since the values did increase during subsequent weeks, the readings at 1 week did not appear to offer a suitable basis for a valid comparison. On the other hand, some of the animals in the faster regenerating groups had already reached the normal range of hemoglobin values by the end of the second week. Consequently, the interpolated value for 1.5 weeks appeared to be the most sensitive point for comparison. When some later point on the curve is used, the quantitative superiority of the more highly

available forms may not be so marked or indeed may no longer be evident. With minor exceptions, the general trend of the results at 1.5 weeks is confirmed by the curves for other points during the test. The marked quantitative superiority of ferrous sulfate iron over ferric orthophosphate iron is readily apparent at the various levels of enrichment.

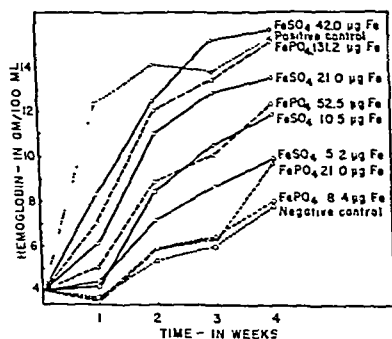


Figure 1

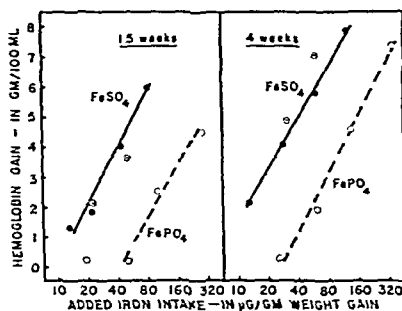


Figure 2

Fig. 1 Hemoglobin regeneration curves of anemic rats fed the indicated levels of ferrous sulfate iron (—) or ferric orthophosphate iron (---), as supplied by enriched bread. Negative and positive control curves are also shown. All points were corrected to an initial hemoglobin value of 4.0 gm/100 ml of blood.

Fig. 2 Dosage response curves at 1.5 weeks and at 4 weeks of anemic rats fed ferrous sulfate iron or ferric orthophosphate iron, as supplied by enriched bread. The ferric chloride points are shown by the circled dots.

The initial hemoglobin values, as listed in table 3, show that the various test groups were at approximately the same degree of anemia at the start of the experiment. The mean hemoglobin gains and iron intakes after 1.5 weeks and 4 weeks are also given in table 3. These data were used to arrive at a definite figure for the comparative availability of ferrous sulfate and ferric orthophosphate in the following way. The total hemoglobin gains were corrected for the hemoglobin gain of the negative control (group 1), and the total iron intakes were corrected for the iron intake due to the diet itself without added iron (11.9 µg/gm). This permitted calculation of hemoglobin gain per µg of added iron intake. The added iron intakes were also corrected for the minor differences in

TABLE 3

*Responses of rats to various sources and levels of iron.*

GROUP	BREAD Compound added	NO. RATS	Iron added $\mu\text{g/gm}$	INITIAL WT. (AV.)		WT. GAIN (AV.)		FOOD CONSUMPTION (AV.)		IRON INTAKE (AV.)		INITIAL HEMOGLOBIN (AV.)		HEMOGLOBIN GAIN MEAN $\pm$ S.E.	
				1.5 wks.	4 wks.	1.5 wks.	4 wks.	1.5 wks.	4 wks.	1.5 wks.	4 wks.	1.5 wks.	4 wks.	1.5 wks.	4 wks.
				gm	gm	gm	gm	gm/day	gm/day	$\mu\text{g/day}$	$\mu\text{g/day}$	gm/100 ml	gm/100 ml	gm/100 ml	$\mu\text{m}/100\text{ ml}$
1	None (negative control)	9		96	35	94	94	9.8	11.3	117	134	3.93	0.48 $\pm$ 0.26	3.69 $\pm$ 0.33	
2	Ferric orthophosphate	8	8.4	84	36	90	90	9.4	12.3	177	231	3.90	0.69 $\pm$ 0.32	3.99 $\pm$ 0.44	
3	Ferric orthophosphate	8	21.0	100	34	90	90	9.1	11.4	266	333	4.01	0.68 $\pm$ 0.33	5.64 $\pm$ 0.64	
4	Ferric orthophosphate	9	52.5	92	45	111	105	10.5	12.9	576	708	3.86	2.96 $\pm$ 0.51	8.31 $\pm$ 0.57	
5	Ferric orthophosphate	10	131.2	98	46	106	106	11.8	12.9	1389	1518	3.88	4.99 $\pm$ 0.76	11.11 $\pm$ 0.41	
6	Ferrous sulfate	9	5.25	94	39	104	104	10.9	11.7	176	190	3.87	1.79 $\pm$ 0.14	5.84 $\pm$ 0.22	
7	Ferrous sulfate	9	10.5	101	46	106	106	11.3	12.2	232	250	3.99	2.30 $\pm$ 0.37	7.82 $\pm$ 0.63	
8	Ferrous sulfate	10	21.0	84	44	100	100	10.5	12.2	307	356	3.57	4.57 $\pm$ 0.39	9.48 $\pm$ 0.63	
9	Ferrous sulfate	9	42.0	93	46	96	96	11.0	12.9	495	580	3.81	6.43 $\pm$ 0.28	11.62 $\pm$ 0.51	
10	Ferric chloride	9	10.5	93	38	85	85	9.2	11.1	188	228	4.17	2.64 $\pm$ 0.45	8.62 $\pm$ 0.83	
11	Ferric chloride	10	21.0	80	36	89	89	9.5	11.3	277	330	3.85	4.09 $\pm$ 0.29	10.77 $\pm$ 0.34	
12	Ferrous sulfate (positive control)	7	244.0	84	45	101	101	8.7	11.1	1843	2352	3.39	9.23 $\pm$ 0.75	11.53 $\pm$ 1.8	

the average weight gains of the groups, although such correction did not modify the final conclusions. This permitted calculation of hemoglobin gain per  $\mu\text{g}$  of added iron intake per gm of weight gain. The resultant dosage response values, illustrated graphically in figure 2, were then compared statistically by the method of Waddell and Kennedy ('47).

At 1.5 weeks ferric orthophosphate iron was  $19.6 (\pm 2.4)\%$  (mean  $\pm$  S.E.) as available as ferrous sulfate iron if the lowest ferric orthophosphate value (group 2) is omitted from the calculation. This may be done on the grounds that the latter point appears to be below the sensitive portion of the dosage response curve. However, comparison of the 2 iron sources without omitting the group 2 value does not change the result markedly, though the standard error does not give so true a picture of the agreement of the data. Calculated to include all points at 1.5 weeks, ferric orthophosphate iron was  $21.2 (\pm 6.8)\%$  as available as ferrous sulfate iron.

At 4 weeks ferric orthophosphate iron was  $25.2 (\pm 2.0)\%$  as available as ferrous sulfate iron. As indicated previously, the latter time does not really give a valid comparison, but it has been included to demonstrate the marked differences in availability which exist even at this less sensitive point.

The excellent agreement between the groups fed differing amounts of the same added iron source is demonstrated in figure 2. Aside from the 1.5-week value for group 2, as noted above, the points fall very nearly on straight lines.

The statistical significance of the differences between the individual groups was determined by calculation of the standard error of the difference between the mean hemoglobin gains both at 1.5 weeks and at 4 weeks, comparisons being made for all groups. A relatively high criterion of significance was adopted by basing conclusions only on P values of 0.01 or less (i.e., probability of the difference being fortuitous equals 1 in 100, or less). The results of this analysis showed that the previously mentioned differences in mean hemoglobin gains between the ferric orthophosphate and ferrous sulfate groups were highly significant. For instance, at 1.5 weeks group 7

(FeSO<sub>4</sub> iron 10.5 µg/gm) was significantly superior ( $P=0.005$ ) to group 3 (FePO<sub>4</sub> iron 21 µg/gm), and was not significantly different ( $P=0.3$ ) from group 4 (FePO<sub>4</sub> iron 52.5 µg/gm). Likewise, group 9 (FeSO<sub>4</sub> iron 42 µg/gm) was greatly superior ( $P=<0.001$ ) to group 4 (FePO<sub>4</sub> iron 52.5 µg/gm), although not significantly superior ( $P=0.09$ ) to group 5 (FePO<sub>4</sub> iron 131.2 µg/gm). The results of the analysis at 4 weeks were almost as significant.

TABLE 4

*Rates of hemoglobin regeneration, based upon days required to reach hemoglobin value of 10 gm per 100 ml.*

GROUP	BREAD		DAYS	RATE OF HEMOGLOBIN REGENERATION AS PERCENTAGE OF OPTIMUM
	Compound added	Iron added µg/gm		
1	None (negative control)	. .	63.0	0
2	Ferric orthophosphate	8.4	47.4	3
3	Ferric orthophosphate	21.0	37.8	6
4	Ferric orthophosphate	52.5	20.5	18
5	Ferric orthophosphate	131.2	10.7	42
6	Ferrous sulfate	5.25	28.7	11
7	Ferrous sulfate	10.5	18.9	21
8	Ferrous sulfate	21.0	12.4	36
9	Ferrous sulfate	42.0	9.8	47
10	Ferric chloride	10.5	20.1	19
11	Ferric chloride	21.0	13.3	33
12	Ferrous sulfate (positive control)	244.0	5.4	100

The results were also calculated in terms of the number of days required to reach an average hemoglobin value of 10 gm/100 ml, which is close to the normal range (see table 4). The various groups were then rated for percentage of optimal hemoglobin regeneration by comparison with the positive control, group 12, which contained a known excess of ferrous sulfate. A correction was made for the hemoglobin regeneration contributed by the iron present in the diet without iron enrichment (group 1, negative control bread). By this method of calculation, also, it was demonstrated that the ferrous sul-



fate iron was approximately 4 to 6 times as effective as ferric orthophosphate iron. Statistical treatment of these data by the previously mentioned procedure (Waddell and Kennedy, '47) showed ferric orthophosphate iron to be  $17.2 (\pm 6.2)\%$  as available as ferrous sulfate iron. This is in general agreement with the comparison based on hemoglobin regeneration at 1.5 weeks.

As shown in table 3, all of the groups of rats grew well. The weight gains of the various groups differed little and thus were in marked contrast to the wide variations in hemoglobin regeneration. Likewise, the differences in food consumption were small, corresponding generally to the small differences in weight gains, and could not account for the large differences in hemoglobin regeneration. In addition, several ferrous sulfate rats were pair-fed with ferric orthophosphate rats to maintain the same individual food consumption. The ferrous sulfate animals again proved much superior in hemoglobin regeneration. A comparison of iron intakes and hemoglobin gains (see table 3) emphasizes the superiority of the biological availability of ferrous sulfate iron over that of ferric orthophosphate iron.

#### *Ferrous sulfate and ferric chloride*

The comparison of ferrous sulfate and ferric chloride at 2 levels showed these forms of iron to be equally effective for regeneration of hemoglobin. The curves for the 2 compounds are practically superimposable, as may be seen in figure 3. Further evidence of similarity is presented by the hemoglobin gains in table 3 and the hemoglobin regeneration rates in table 4. Calculations were made for the standard error of the difference between the mean hemoglobin gains. The analysis at 1.5 weeks showed that there was no significant difference ( $P=0.56$ ) between group 7 ( $\text{FeSO}_4$  iron  $10.5 \mu\text{g/gm}$ ) and group 10 ( $\text{FeCl}_3$  iron  $10.5 \mu\text{g/gm}$ ). Similarly, there was no significant difference ( $P=0.32$ ) between group 8 ( $\text{FeSO}_4$  iron  $21 \mu\text{g/gm}$ ) and group 11 ( $\text{FeCl}_3$  iron  $21 \mu\text{g/gm}$ ). The analysis at 4 weeks likewise demonstrated that there was no significant

difference between the ferrous sulfate and ferric chloride values. The highly available ferric chloride iron, like that of ferrous sulfate, was 4 to 5 times as effective as ferric orthophosphate iron (see fig. 2). This study indicated that results based on ferric chloride standards should be directly comparable with those based on ferrous sulfate standards.

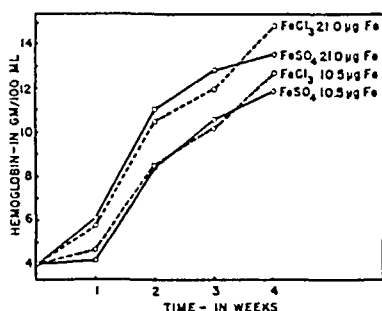


Fig. 3 Hemoglobin regeneration curves of anemic rats fed similar levels of ferrous sulfate iron (—) and ferric chloride iron (---), as supplied by enriched bread. All points were corrected to an initial hemoglobin value of 4.0 gm/100 ml of blood.

## DISCUSSION

Several experiments have now been reported on the utilization of ferric orthophosphate iron in rats. Although Day and Stein ('38) did not attempt a truly quantitative comparison, their results in a single-level study indicated that ferric chloride iron was approximately 4 times as available as ferric orthophosphate iron. In their single experiment, Freeman and Burrill ('45) found ferric orthophosphate iron to be practically as effective as ferric chloride iron. Inasmuch as Freeman and Burrill report only the final hemoglobin values at the end of 28 days, a time at which the groups were already well within the normal hemoglobin range, it is possible that differences occurring at 1 to 2 weeks were no longer apparent. In 2 previous single-level experiments, Blumberg and Arnold ('47) found ferrous sulfate iron to be more than twice as available as ferric orthophosphate iron. The present investigation at multiple levels appears to leave little doubt that, under the

conditions of these experiments, ferrous sulfate iron or ferric chloride iron is approximately 4 to 5 times as available for hemoglobin regeneration as is ferric orthophosphate iron. It may be noted that McCance et al. ('43) observed in human subjects that the addition of disodium phosphate to bread decreased the absorption of iron. Caution must be exercised against confusing ferric orthophosphate itself with preparations in which the ferric orthophosphate has been solubilized with sodium citrate, e.g., Soluble Ferric Phosphate, N. F. VIII (National Formulary, '46).

The magnitude of the enrichment program justifies a thorough appraisal of the assimilability of the iron sources in use or proposed for use. Although the results of rat experiments are not applicable to man with certainty, these investigations with enriched bread strongly suggest the advisability of seeking better sources of iron than ferric orthophosphate for bread enrichment. The clinical findings of Moore and coworkers ('39) suggest that in man also ferric orthophosphate is poorly utilized as compared with ferrous sulfate. Certainly further studies upon the efficacy of ferric orthophosphate in both animals and man should be conducted if its use is to be continued. From a nutritional standpoint it would appear safer to use an iron source already known to be highly efficacious in man, such as ferrous sulfate, reduced iron, or other preparations of comparable availability. In order that the consumer may secure the full benefit of the enrichment program, it is desirable that highly assimilable forms of iron be used in bread and flour enrichment.

#### SUMMARY

The biological availabilities of the iron in ferrous sulfate and ferric orthophosphate have been compared on the basis of hemoglobin regeneration in rats made anemic from iron deficiency. A secondary comparison was made with ferric chloride. The iron compounds were fed in the form of enriched breads, and multiple levels of iron enrichment were used to permit comparison of dosage response curves.

Under the conditions of these experiments, ferrous sulfate iron was 4 to 5 times as available as ferric orthophosphate iron when both compounds were tested at 4 widely spaced levels.

When compared at 2 levels, ferric chloride iron was equal in biological availability to the highly effective ferrous sulfate iron, or 4 to 5 times as available as ferric orthophosphate iron.

Attention is called again to the desirability of using highly assimilable forms of iron in flour and bread enrichment, so that the consumer may secure the full benefit of the enrichment program.

#### ACKNOWLEDGMENT

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# MODIFICATION OF THE ACTION OF CAFFEINE ON THE SPONTANEOUS ACTIVITY OF THE WHITE RAT BY THE CONCOMITANT ADMINISTRATION OF VARIOUS FOOD MATERIALS<sup>1, 2</sup>

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## SEVEN FIGURES

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Various investigators have found that caffeine above a certain threshold level has a stimulating action on the central nervous system (Hollingworth, '12; Cheney, '35; Horst and Jenkins, '35).

The present experiments are offered to show that this action of caffeine as manifested by an increase in the spontaneous activity of the albino rat, can be modified to an appreciable extent by certain food materials administered either simultaneously with the administration of caffeine or a short time afterward.

## PROCEDURE

The method used for measuring spontaneous activity was that described by Schulte and his associates ('41). The apparatus consisted of a small wire cage measuring 6" × 6" × 6" which was suspended from a spiral spring in such a manner

<sup>1</sup> Preliminary report, *Fed. Proc.*, 6: 119, 1947.

<sup>2</sup> The expense of this investigation was defrayed in large part by a grant-in aid from the Sugar Research Foundation.

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that all the movements of the animals, even the slightest ones, were summated by a work adder attached to the spring. A small pin inserted in the rim of the work adder dipped into a mercury cup upon each complete revolution thereby completing an electric circuit and activating a signal magnet writing on a slowly moving kymograph paper. Activity was measured and recorded in terms of the total number of revolutions of the work adder throughout the experiment. Twelve units which were set up in a small well-lighted room devoted exclusively at the time to the experiment were used simultaneously. All the experiments were run in the morning because at this time of day in a well-lighted room the activity of a white rat is at a minimum.

Seven sets of observations were made under different experimental conditions. Caffeine alkaloid in various amounts was administered in aqueous solution by stomach tube or intraperitoneally. In some experiments sugar dissolved in water was given by stomach tube simultaneously with or shortly after the administration of caffeine. In others peptone, vegetable oil, and the inert material agar were given instead of sugar. Further details of the procedure will be described under the following separate captions.

#### RESULTS

*Experiment 1: Showing that caffeine causes an increase in spontaneous activity for several hours after administration whereas no permanent effect is produced by the daily consumption of a large amount of caffeine*

Twenty-two animals were selected 1 week after weaning and given 75 mg caffeine/kilo daily in aqueous solution by stomach tube for 100 days. An equal number of litter mates were chosen for controls and by the same procedure given water daily instead of the caffeine solution. At the end of this period 24 hours were allowed to elapse without the administration of caffeine whereupon the animals were placed



in the activity cages for the first time. They were kept in the cages for 1 hour for the purpose of obtaining their basal activity, then removed and given caffeine or water by stomach tube and replaced in the cages. This procedure was repeated on successive days (except on Sundays) until 220 daily observations had been made on the test animals and the same number on the controls.

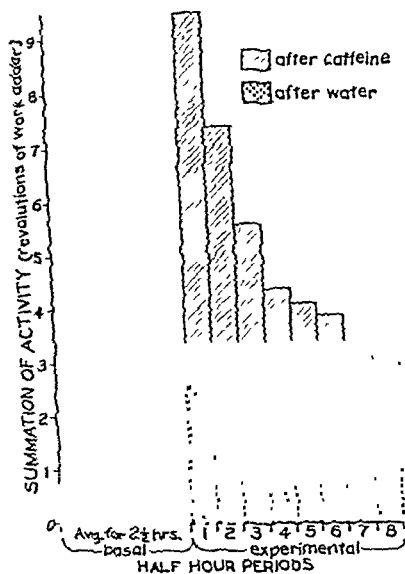


Figure 1

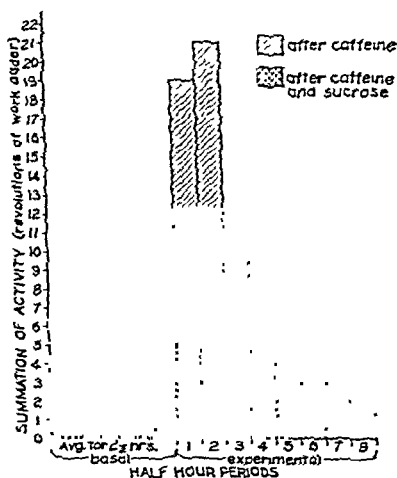


Figure 2

Fig. 1 The effect of 75 mg caffeine/kg on spontaneous activity. For the sake of continuity the activity of the animals under test and control conditions is represented by hatched and cross-hatched lines, respectively, although caffeine was not given to the test animals nor water to the controls until the end of the basal period.

Fig. 2 Comparison of spontaneous activity after caffeine in water and caffeine in a sucrose solution.

Seventy-five mg caffeine per kg body weight had a definite stimulating effect on spontaneous activity which was most marked during the first half hour. This effect was still manifest at the conclusion of the experiment 4 hours after the administration of caffeine. The results of the experiment are presented in figure 1. The activity of the test animals was

greater than that of the controls in 206 out of 220 individual experiments; in 5 experiments there was difference whereas in 9, the controls were slightly more active than the test animals.

Long continued daily administration of this amount of caffeine had no permanent effect as shown by the fact that the basal activity of the animals that had been given caffeine for 100 days was the same as that of the controls. In this connection it is of interest to note that in human subjects Cheney ('35) found no significant effect on reaction time 24 hours after the consumption of caffeine.

*Experiment 2: Showing that sucrose administered simultaneously with caffeine reduces the increase in spontaneous activity caused by the stimulating action of caffeine*

These experiments were undertaken with the purpose in mind of following up a lead obtained in other observations (not published) which suggested that the action of caffeine might be affected by the simultaneous ingestion of sugar.

*In this and the following experiments each animal served as its own control. The animals were divided into 2 groups. On the first day of the experiment 1 group was studied under experimental and the other under control conditions. On alternate days thereafter this order was reversed.*

Twelve adult rats were selected at random from our colony. Under control conditions the animals were given 20 mg caffeine/kg in aqueous solution by stomach tube and under test conditions the same amount of caffeine in a 50% sucrose solution (1.5 gm sucrose/100 gm body weight). It had been found previously in another set of experiments that 20 mg caffeine/kg had a definite stimulating action. These results are not presented separately as they are in agreement with those obtained with this amount of caffeine in the experiments shown in figure 2. Each point on the nomogram is plotted from an average of 60 experiments.

A comparison of the activity when caffeine alone was given with that which occurred after the simultaneous administration of caffeine and sucrose (fig. 2) shows that the sugar neutralized to a large extent the action of caffeine.

In other experiments, the results of which need not be given in detail, spontaneous activity after the administration of a sucrose solution alone was found to be practically the same as when plain water was given.

*Experiment 3: Showing that the increase in activity caused by caffeine can be reduced by administering sucrose 30 minutes after giving caffeine*

When it had been definitely shown in the preceding experiments that the ingestion of sucrose had a modifying effect on the action of caffeine, speculation arose as to the mechanism involved. To determine whether the effect of the simultaneous administration of sugar might be due to an interference with the absorption of caffeine the following experiments were undertaken.

The animals used in this experiment were the same as in experiment 2. The procedure and the amount of caffeine and sucrose administered were also the same with the exception that all the animals were given caffeine immediately after the basal period. Thirty minutes later those under test conditions were given the sucrose solution by stomach tube and those under control conditions were given the same amount of water. There were 48 observations under each set of conditions.

The area of cross-hatched lines in figure 3 for the first half hour after the basal period represents the activity of the animals on the days when they were given sucrose after this first half hour period; the hatched lines, the activity on the days when they were given water (control experiments). Within 30 minutes the caffeine had been absorbed and was exerting its maximum effect, as indicated by the activity of the animals at this time. Administration of sucrose was fol-

lowed in the next half hour period by a definitely greater decrease in activity than when water alone was given.

*Experiment 4: Showing that caffeine injected intraperitoneally increases spontaneous activity which in turn is reduced by the oral administration of sucrose*

This experiment is offered as confirmatory evidence that the effect of sucrose on the action of caffeine is not due to an interference with absorption. The observations were made on the same animals as in the preceding experiment.

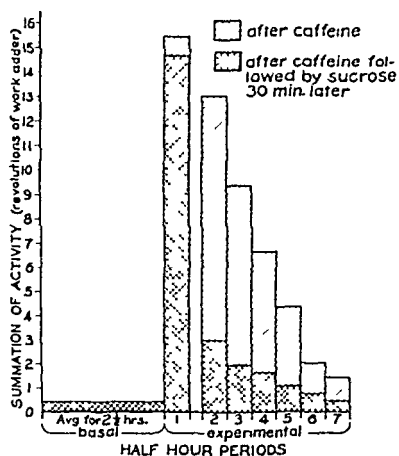


Figure 3

Fig. 3 Reduction in the increased activity produced by caffeine when sucrose was administered 30 minutes after caffeine.

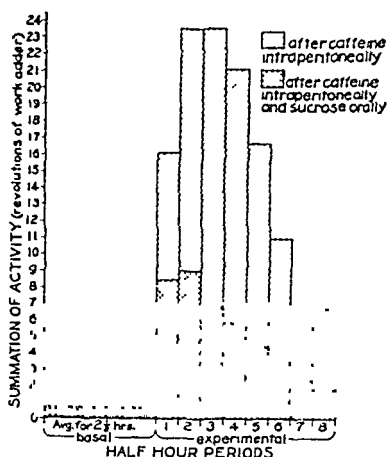


Figure 4

Fig. 4 The action of the oral administration of sugar on the increase in spontaneous activity caused by the intraperitoneal injection of caffeine.

An aqueous solution containing 20 mg caffeine/kg was injected intraperitoneally into all the animals at the conclusion of the basal period. Immediately after the injection 1 group was given sucrose by stomach tube and the other water only. On alternate days this procedure was reversed.

The average values of the data obtained are plotted in figure 4. Each average was derived from 36 experiments.

During the first half hour after intraperitoneal injection of caffeine the activity was practically the same as in the preceding experiments in which caffeine was administered by stomach tube. In the second half hour there was a further increase in activity which continued at this high level during the following half hour period. From this time on there was a gradual return toward the basal level.

The oral administration of sugar neutralized to a large extent the stimulating action of caffeine that had been injected intraperitoneally.

*Experiment 5: Showing that the increase in spontaneous activity induced by caffeine is reduced by the simultaneous administration of peptone*

Further speculation on the mechanism whereby sucrose modifies the action of caffeine suggested the advisability of determining whether this effect was specific with respect to sucrose or common to other food materials of an entirely different nature. This experiment and experiment 6 were designed for this purpose.

Twelve animals that had not been used in the previous experiments were selected from the colony. The procedure was the same as in experiment 2 except that an aqueous solution containing 10 gm peptone/kg body weight was given instead of the sucrose solution.

The results presented in figure 5 show that peptone had an effect similar to that of sucrose in reducing the increase in activity caused by caffeine.

*Experiment 6: Showing that administration of vegetable oil simultaneously with caffeine reduces the increased activity resulting from caffeine*

Results similar to those in experiment 5 were obtained in the present experiment in which 20 mg caffeine dissolved in 3.75 ml vegetable oil was administered by stomach tube to 12 animals. The comparative effects of caffeine in water and

caffeine in oil are presented in figure 6. Each value from which the nomogram was constructed is an average of 60 experiments.

From this experiment and the preceding one it is apparent that the modifying effect on the action of caffeine which was observed originally in our studies with sucrose is not specific for sucrose but is shared by the other major foodstuffs, namely, protein and fat.

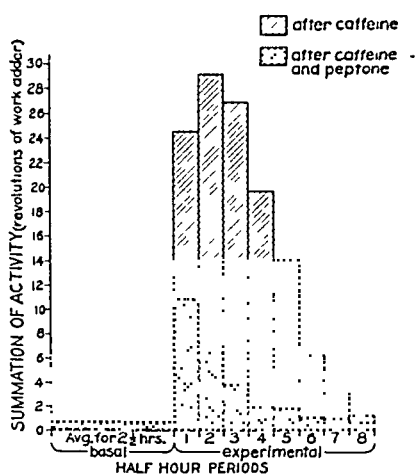


Figure 5

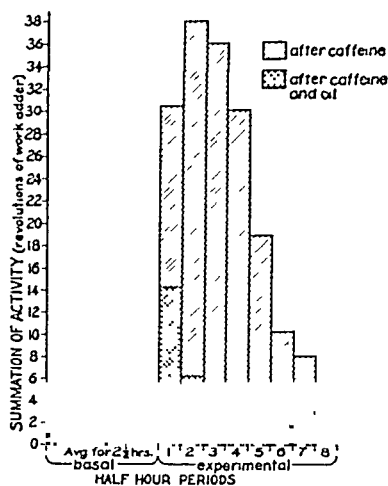


Figure 6

Fig. 5 Reduction in the stimulating action of caffeine by the simultaneous administration of peptone.

Fig. 6 The effect of the simultaneous administration of vegetable oil on the stimulating action of caffeine.

*Experiment 7: Showing that the increase in spontaneous activity caused by caffeine is not affected by the simultaneous administration of inert material*

There remained to be considered the possibility that the mere presence of materials other than caffeine in the gastrointestinal tract, regardless of their nature, might have an effect similar to that produced by the foodstuffs. This experiment, the results of which are presented in figure 7, shows that this is not the case. There was no significant difference in

the activity of the animals when given 20 mg caffeine/kg in aqueous solution by stomach tube and when given the same amount of caffeine dissolved in 10% agar. Each value plotted is an average of 30 observations.

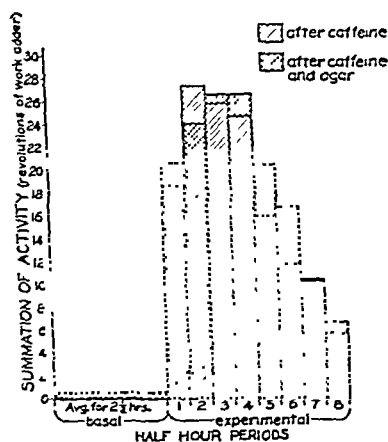


Fig. 7 Spontaneous activity following the administration of caffeine in water and caffeine in agar.

## DISCUSSION

The original suggestion for the pursuit of this investigation arose in the course of another study still in progress which seemed to indicate that certain physiological actions of caffeine might be modified by the simultaneous ingestion of sucrose. The present study is concerned with a different underlying physiological mechanism.

While these experiments were under way it came to our attention that it had been reported by Chaucard ('45) and in another paper by the same author and his associates (Chaucard, Mazoue and Lecoq, '45) that the chronaxie of nerve fibers is affected by caffeine and that this effect is specifically neutralized by the oral administration of the sugars: lactose, sucrose, galactose and glucose.

When it had been proved to our satisfaction that the action of caffeine on the central nervous system can be modified by the administration of sucrose, our interest was aroused in

the mechanisms involved. The only contribution we can offer at the present time with regard to the question is of a negative nature as stated in the summary.

#### SUMMARY

1. The modification of the action of caffeine by sucrose is not specific to this food material but is shared by foods of an entirely different nature, namely, protein and fat.

2. It is not due to an interference with absorption for, as shown in experiments 3 and 4, the effect was observed when caffeine was injected intraperitoneally and also when sugar was given after caffeine had been absorbed and had produced its characteristic action.

3. It cannot be accounted for by the mere presence of other material regardless of its nature in the gastro-intestinal tract, for agar administered simultaneously with caffeine had no neutralizing action.

In respect to the lethal dose of caffeine our results differ from those of Schulte and his associates ('39). A dose of 40 mg caffeine/kg proved fatal in 1 of their 10 animals. Since 10 mg/kg sufficed to increase spontaneous activity it was concluded that the margin of safety between the barely effective and the convulsive or fatal dose was 4 times. In the experiments reported in this paper and in numerous other experiments we have administered 75 mg caffeine/kg over 9000 times to 145 different rats without a single fatality.

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# SELF SELECTION OF DIET

## V. APPETITE FOR CARBOHYDRATES<sup>1</sup>

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### TWO FIGURES

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In a previous experiment, it was found that most young growing rats had little appetite for sucrose, when their choice of food was casein, fat, salt mixture, and sugar (Scott, '46). The present report gives the results of experiments in which other carbohydrates (starch, dextrin and lactose) were offered as choices instead of or in addition to sucrose.

### EXPERIMENTAL

In each of the first 3 experiments, 10 male and 10 female weanling rats were placed in individual cages and allowed to eat a mixed diet<sup>2</sup> offered in all of 4 cups. The experiments differed in that the sources of carbohydrate in the diets were different. Raw corn starch was the carbohydrate in the first experiment, lactose in the second, and dextrin in the third. Vitamins were given separately as pills.<sup>3</sup> During a 3-week

<sup>1</sup>Contribution no. 640 of the Department of Chemistry, University of Pittsburgh. Aided by grants of the Nutrition Foundation, Inc., and the Buhl Foundation.

<sup>2</sup>The mixed diet consisted of 62% carbohydrate, 10% hydrogenated fat (Primex), 4% salt mixture (Jones and Foster, '42), and 24% casein (Labco "vitamin-free").

<sup>3</sup>One pill was given each rat daily. It contained: 60  $\mu$ g thiamine hydrochloride, 120  $\mu$ g riboflavin, 90  $\mu$ g pyridoxine hydrochloride, 150  $\mu$ g calcium pantothenate, 10 mg choline chloride, 1 mg  $\alpha$ -tocopherol, and 55 I.U. vitamin A, and 11 I.U. vitamin D as 0.001 ml Natola; all in a dextrin-powdered sugar base.

control period, the amount eaten from each cup was recorded daily and the cups were then interchanged in a predetermined random manner. During a 3-week experimental period, the choices given the animals were fat, protein, salt mixture, and the same carbohydrate as in the control period, each in a separate cup. The amount eaten was recorded and the cups interchanged daily as before.

In the fourth experiment, 20 male and 20 female weanling rats were divided into 4 groups so that each animal had 1 littermate of the same sex in each of the other 3 groups. The members of each group were fed 1 of the following mixed diets in all of 4 cups during the control period: (1) 62% sucrose diet, (2) 62% starch diet, (3) 62% lactose diet, (4) 62% dextrin diet. In the experimental period all animals were given their choice in 4 separate cups of these 4 mixed diets.

In the final experiment, each of 20 rats was placed in a cage with 6 cups, all of which contained a diet consisting of 24% casein, 10% fat, 4% salt mixture, and 15.5% each of sucrose, starch, lactose and dextrin. During the experimental period 7 choices were allowed the animals. These consisted of casein, fat, salts, sucrose, starch, lactose and dextrin, each in a separate cup.

## RESULTS

The growth and food consumption of the animals during the control period are shown in tables 1 and 2. All of the animals fed lactose diets developed severe diarrhea and grew very slowly. The other diets were approximately but not completely equivalent nutritionally as shown in table 2. The decreasing growth rates with these carbohydrates were: starch > dextrin > sucrose, with the difference between starch and sucrose definitely significant.

The results of the first 3 experiments and the 7-choice experiment are shown graphically in figure 1, while the carbohydrate choices in the latter experiment are shown in figure 2. The results of the experiment where mixed diets were offered are shown in table 3.

TABLE 1

*Growth and food consumption in the control period.<sup>1</sup>*

EXPT	CARBOHYDRATE SOURCE	MALES		FEMALES	
		Wt gain	Food eaten	Wt gain	Food eaten
		gm	gm	gm	gm
1	Starch	104.5 ± 5.0	210.7 ± 10.4	82.7 ± 1.3	178.0 ± 6.2
2	Lactose	30.0 ± 3.3	116.8 ± 5.5	33.4 ± 4.2	110.1 ± 7.8
3	Dextrin	76.5 ± 4.1	190.4 ± 5.8	66.2 ± 3.7	176.1 ± 8.1
5	Mixture	70.7 ± 3.3	176.1 ± 4.6	62.3 ± 2.3	181.6 ± 4.9

<sup>1</sup> All data in terms of mean and standard error of the mean.

TABLE 2

*Growth and food consumption during the control period.*

(Experiment 4)

CARBOHYDRATE SOURCE	WT GAIN	FOOD EATEN
	gm	gm
Sucrose	62.5 ± 3.6	133.0 ± 7.7
Starch	73.9 ± 3.6	161.8 ± 8.8
Lactose	31.2 ± 4.1	103.4 ± 6.0
Dextrin	68.7 ± 3.6	155.6 ± 5.2

TABLE 3

*Preference for mixed diets.*

CARBOHYDRATE SOURCE IN CONTROL PERIOD	WT GAIN	TOTAL FOOD	APPETITE FOR DIET CONTAINING <sup>1</sup>			
			Sucrose	Starch	Lactose	Dextrin
Sucrose	78.7 ± 7.4	248.9 ± 17.2	-19.5 ± 5.7	18.6 ± 7.2	-19.2 ± 3.4	21.2 ± 10.0
Starch	70.5 ± 6.6	246.6 ± 16.3	7.9 ± 6.4	-12.6 ± 7.0	-23.0 ± 2.1	27.0 ± 7.0
Lactose	81.9 ± 3.6	225.6 ± 10.6	-5.9 ± 7.3	-1.0 ± 10.2	-16.9 ± 3.2	23.8 ± 9.7
Dextrin	67.9 ± 5.8	225.3 ± 19.7	16.1 ± 7.0	2.3 ± 9.0	-25.8 ± 5.1	7.2 ± 6.6
Average	74.8 ± 3.0	244.1 ± 6.5	0.4 ± 3.9	1.8 ± 4.4	-21.2 ± 1.8	19.8 ± 4.2

<sup>1</sup> Change in percentage eaten (experimental minus control) from corresponding cups during control and experimental periods.

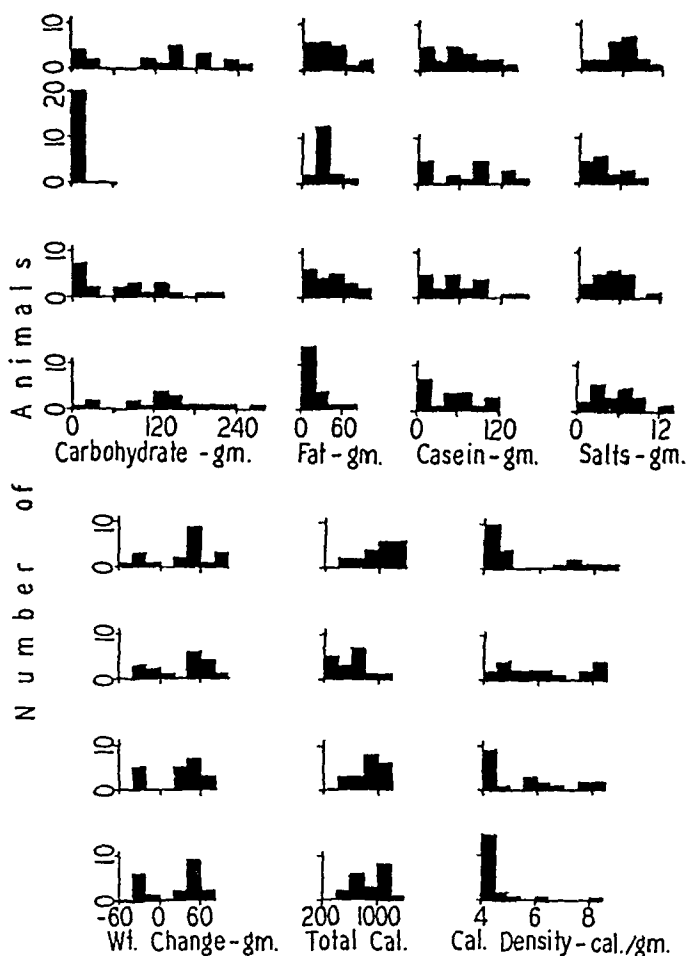


Fig. 1 Weight change and selections of animals when various carbohydrates were offered. The carbohydrate offered was in each case: Top histogram — starch; second histogram — lactose; third histogram — dextrin; lower histogram — sucrose, starch, lactose and dextrin offered simultaneously.

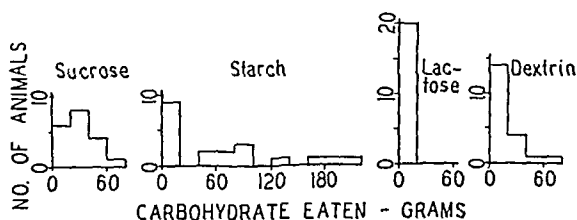


Fig. 2 Selection of carbohydrates when offered simultaneously.

## DISCUSSION

In all of the present experiments lactose was thoroughly disliked. There was no obvious reason for this, but it is not inconceivable that the physiological response to this carbohydrate resulting in diarrhea may have caused unpleasant subjective symptoms in the animal. The other carbohydrates were accepted to a greater or less degree by some animals. The order of preference varied with the type of experiment,

TABLE 4  
*Effect of carbohydrate choice on protein choice.*

CARBOHYDRATE CHOICE	NUMBER OF ANIMALS IN		PROBABILITY <sup>2</sup>
	Group A <sup>1</sup>	Group B	
Sucrose	34	53	1.000
Starch	5	15	0.214
Lactose	7	13	0.777
Dextrin	5	15	0.214
All four	7	13	0.777

<sup>1</sup> Group A ate little or no protein and lost weight; Group B selected protein and gained weight during the experimental period.

<sup>2</sup> Probability that an arrangement into groups as improbable as that shown could occur through random sampling. The theoretical probability of a rat eating protein was taken as that found in the sucrose experiment.

and the following orders of acceptability were observed: 1 carbohydrate as a choice (first 3 experiments), starch > dextrin > sucrose; 4 carbohydrates as choices (fifth experiment), starch > sucrose > dextrin; mixed diets as choices, dextrin > starch = sucrose.

The variation in intake of carbohydrate was accompanied by correlated inverse variation in fat. This is shown particularly well in the caloric density histograms, where it is seen that in those experiments where large amounts of carbohydrate were selected, the caloric density of the selections was low. Variation in carbohydrate intake had no observable effect on the number of animals which accepted protein as a choice, as shown in table 4. However, the complete avoidance

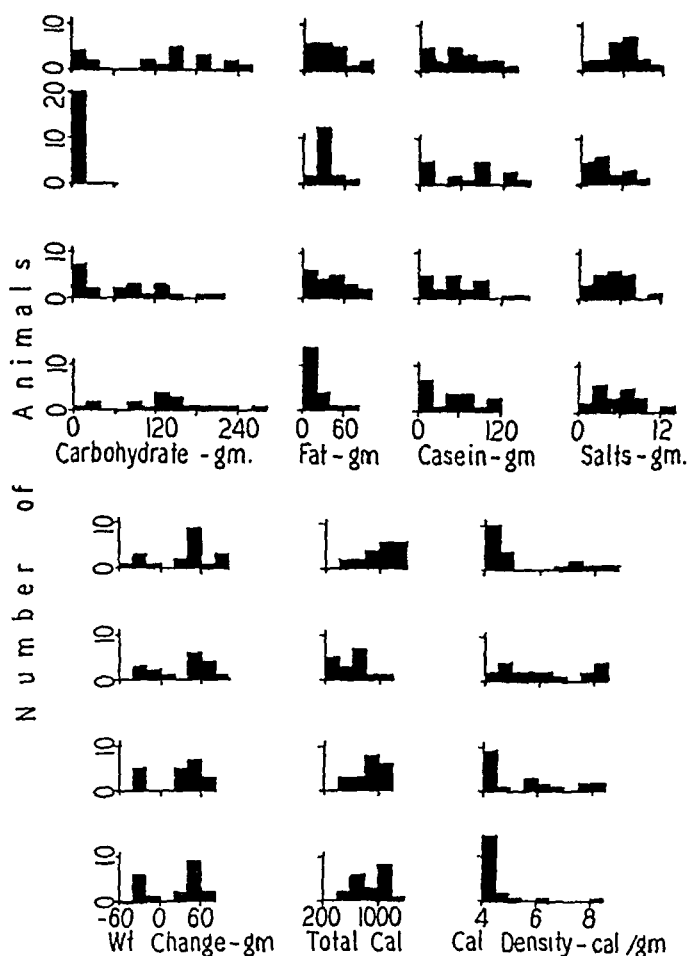


Fig. 1 Weight change and selections of animals when various carbohydrates were offered. The carbohydrate offered was in each case: Top histogram — starch; second histogram — lactose; third histogram — dextrin; lower histogram — sucrose, starch, lactose and dextrin offered simultaneously.

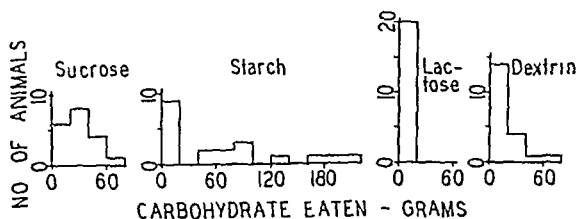


Fig. 2 Selection of carbohydrates when offered simultaneously.



## SUMMARY

Of 4 carbohydrates tested in an experiment where rats were allowed to choose the components of a diet, lactose was avoided, but sucrose, starch, and dextrin were accepted to various degrees. The appetites for various carbohydrates were concluded to be largely independent of appetite for casein, inversely proportional to the intake of fat, independent (except that for lactose) of nutritional significance, dependent on the method of offering the choice, and independent of each other. Rats appeared to relish a change in the carbohydrate portion of their ration.

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of lactose did result in some cases in high intakes of casein. This is apparent from the protein histogram, where it is seen that several animals ate large amounts of casein, in spite of the fact that, being smaller animals, their caloric intake was rather low.

For the most part, then, protein intake was independent of carbohydrate intake, but fat intake was intimately related to it. The 3 carbohydrates other than lactose were approximately equivalent nutritionally, and yet were accepted to different extents, and acceptance was dependent in part on how the choices were offered. It is improbable, therefore, that selection in these 3 cases was based on any nutritional qualities of the choices, and probable that the appetites shown for these substances were trivial in origin.

Furthermore, it appeared incorrect to speak of an "appetite for carbohydrate" since the appetites studied were essentially independent. The probability based on observation that an animal would not eat at least 20 gm of a given carbohydrate was as follows: sucrose 0.50; starch 0.20; lactose 1.00; dextrin 0.30. Assuming that the appetites were independent, the probability that an animal would not eat at least 20 gm of all 4 would be expected to be the product of the observed probabilities, or 0.03. Actually, only 1 out of 20 rats ate less than 20 gm of carbohydrate in the 7-choice experiment. The observed fraction was then 0.05, in close agreement with the expected probability.

The effect of previous diet on choice can scarcely be decided *a priori*, since one could as well expect animals to continue to eat the diet to which they are accustomed as to expect them to desire variety. It has been our qualitative impression from several experiments that rats relish a change of diet, although the effect only lasts for a day or two. This impression appeared to be substantiated by the data in table 3, where, in all cases but lactose, that group which liked a certain choice least was the group previously fed that choice.

## SUMMARY

Of 4 carbohydrates tested in an experiment where rats were allowed to choose the components of a diet, lactose was avoided, but sucrose, starch, and dextrin were accepted to various degrees. The appetites for various carbohydrates were concluded to be largely independent of appetite for casein, inversely proportional to the intake of fat, independent (except that for lactose) of nutritional significance, dependent on the method of offering the choice, and independent of each other. Rats appeared to relish a change in the carbohydrate portion of their ration.

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# PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

## IX. INFLUENCE OF ASCORBIC ACID STABILIZERS IN FRUITS AND VEGETABLES<sup>1</sup>

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In many homes fruit juices low in ascorbic acid are used interchangeably with those of higher ascorbic acid content. Some nutritionists have recommended that the former products be fortified with the vitamin to make them nutritionally more comparable with the higher potency juices. During and since the war, fortification of apple juice with ascorbic acid (35 mg per 100 ml) has been sponsored by the Canadian government (McFarlane and Davis, '41). In 1943-1945 the U. S. Army produced over 50 million pounds of canned apple juice in Australia, of which about 90% was fortified with synthetic ascorbic acid (Esselen, Powers and Fellers, '46). Enriched apple juice is now available on the American market. Procedures for the fortification of apple juice with ascorbic acid in commercial practice have recently been described by Hoffmann-LaRoche ('46), Merck ('46) and Pfizer ('46). In all these cases it has been the objective to obtain a fruit juice furnishing the minimum daily requirement, 30 mg

<sup>1</sup>The data in this report were presented before the American Institute of Nutrition, 1947, Chicago, Illinois. The expenses of the study were defrayed by a grant from the Duffy-Mott Company, Inc., New York, N. Y.

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of ascorbic acid (Federal Food, Drug and Cosmetic Act, '41) per serving of 100 or 120 ml. Furthermore, the vitamin has been reported to have a favorable effect on color retention of the fruit juice during storage (Esselen, Powers and Fellers, '46).

Interest in the vitamin C contribution of fortified apple juice was markedly stimulated by the recent report by Crampton and Burton ('46) that the vitamin potency is 35% greater than ascorbic acid content. These authors employed a new biological assay procedure, in which the maximum development in height of the odontoblast cells of the incisor teeth of young guinea pigs was the criterion of potency. Either of 2 factors could be responsible for the greater than theoretical values obtained; (a) an unknown factor in apple juice may act synergistically with ascorbic acid, permitting optimal absorption and utilization of the vitamin, or (b) the biological procedure is not specific for ascorbic acid, and some other compound naturally present in the juice exhibits a similar effect.

Studies suggesting that a mixture of flavone glycosides (citrin, or vitamin P) is required for the cure of certain hemorrhagic manifestations of scurvy have recently been summarized (Nutrition Reviews, '43, '44). Much of the evidence is based upon observations that natural foods rich in vitamin C are far more effective in the treatment of hemorrhagic conditions than equivalent amounts of synthetic ascorbic acid. Citrin, together with ascorbic acid, appears to be required for the maintenance of the integrity of the capillary wall.

On the other hand, it has been postulated (Elmby and Warburg, '37) that natural foods may contain an unknown factor, a covitamin, required for more effective utilization of ascorbic acid. The recent report by Crampton and Burton ('46) supports this hypothesis since the authors consider their newly developed bioassay procedure to be specific for ascorbic acid. They found that fruit juices (orange, apple and tomato) exhibit about 35 to 45% higher vitamin C potency than is

indicated by chemical assay of these materials, assays which included determination of dehydro- as well as reduced ascorbic acid. The reference material in the bioassays was the synthetic vitamin. That natural foods contain factors which inhibit oxidative loss of ascorbic acid has been reported by Somogyi ('45) and Reder ('46). The latter suggests that compounds containing the sulfur groups, -SS- and -SH-, in vegetable extracts induce greater retention of ascorbic acid when comparisons are made with the results of control tests of pure solutions of ascorbic acid (buffered or unbuffered) at the same pH value.

Studies of factors influencing the degree of availability of ascorbic acid from different sources have appeared in the recent literature. Tests with humans have shown that ascorbic acid oxidase, capable of rapid *in vitro* destruction of large quantities of vitamin C, is inactive in the digestive tract (Hochberg, Melnick and Oser, '45a). Apparently the enzyme is destroyed *in vivo* or its activity inhibited. Likewise the destructive effect of copper on ascorbic acid is not manifest in the digestive tract (Hochberg, Melnick and Oser, '45b). Thus, the importance of these 2 factors which could conceivably have been responsible for low values for available ascorbic acid can now be discounted. On the other hand, the possibility that ascorbic acid derived from fruits and vegetables exhibits a greater potency than the vitamin in pure solution still requires investigation. As the result of earlier studies it has been concluded that the ascorbic acid in orange juice (Hawley, Stephens and Anderson, '36), in raspberries (Todhunter and Fatzer, '40), in potatoes (Clayton and Folsom, '40), in cabbage and tomato juice (Clayton and Borden, '43), and in papayas and guavas (Hartzler, '45) is available to the human organism to the same degree as the synthetic vitamin in pure solution. However, in these investigations the basal dietaries were not free from other fruits and vegetables. The objective was to reduce the irrelevant ascorbic acid content to a minimum: despite this the dietaries still contributed from 5 to 20 mg of the vitamin per day. In view of the obser-

vation that certain vegetable extracts, even those low in ascorbic acid content, may contain vitamin C stabilizers (Somogyi, '45; Reder, '46) and since these may be responsible for enhancement of biological potency, it would seem quite likely that in the earlier studies the synthetic ascorbic acid taken with the basal dietary may also have been protected by the stabilizing factors present in the vegetables and fruits consumed. It is pertinent to point out in this connection that the protective factor in vegetables is not destroyed by boiling the extracts (Reder, '46).

The objectives of the study now being reported were to answer these questions: (1) is apple juice a favorable vehicle for ascorbic acid as a means of fortifying the human dietary, and (2) what influence do the stabilizing factors in fruits and vegetables have on ascorbic acid availability?

#### EXPERIMENTAL PART

##### *In vitro study*

That fruit juices contain ascorbic acid stabilizing factors has been demonstrated by the *in vitro* tests presented in table 1.

The analyses for ascorbic acid content were conducted according to the photometric procedure (Hochberg, Melnick and Oser, '43). Acidulated, but not buffered, deionized water was employed in the first test system. The second consisted of pasteurized apple juice at its natural pH, and the third of freshly expressed orange juice also at its natural pH. The ascorbic acid was added to the acidulated water and to the apple juice in amounts comparable to that found in the orange juice. The solutions were neither deaerated prior to fortification nor kept under nitrogen during solution of the vitamin. The samples were stored in cylindrical glass-stoppered jars under air with a head space of about one-tenth of the total capacity. At the end of 10 and 21 days' storage of the solutions at 45°C., 1 jar in each series was removed for analysis.<sup>4</sup> The

<sup>4</sup>No changes in pH occurred during storage of the samples.



values reported in table 1 are for total ascorbic acid, since very little dehydroascorbic acid was found. It will be noted from the results of these accelerated holding tests that the vitamin was appreciably more stable in orange juice than in apple juice, while in the latter, retention of ascorbic acid was far superior to that in pure aqueous solution.

TABLE 1  
*Stability of ascorbic acid in solution.*

TEST SYSTEM <sup>1</sup>	TOTAL ASCORBIC ACID CONTENT			RETENTION OF ASCORBIC ACID	
	Freshly prepared <sup>2</sup>	Stored 10 days at 45°C.	Stored 21 days at 45°C.	Stored 10 days at 45°C.	Stored 21 days at 45°C.
	mg/100 ml	mg/100 ml	mg/100 ml	%	%
Aqueous <sup>3</sup> solution of ascorbic acid at pH 3.35 (unbuffered)	56.0	7.3	0.3	13	0.5
Apple juice fortified with ascorbic acid, pH 3.30	51.4	34.3	4.6	67	9
Orange juice, freshly prepared, pH 3.40	56.1	48.3	13.4	86	24

<sup>1</sup> Ascorbic acid was added to the acidulated water and to the apple juice in amounts comparable to that naturally present in the orange juice. The solutions were neither deaerated prior to fortification nor under nitrogen during solution of the vitamin and subsequent storage. In each case (including the orange juice) the air head-space above the solutions in the cylindrical glass-stoppered jars was about one-tenth of the total volume.

<sup>2</sup> The solutions were held for a period of 2 hours at room temperature prior to the first series of assays.

<sup>3</sup> Deionized water.

### *In vivo studies*

In line with the first objective of this investigation, an assay was conducted with human subjects to determine the availability of ascorbic acid in fortified apple juice in comparison with the availability of the vitamin when supplied by raw fruits and vegetables. Ascorbic acid oxidase in the latter products has already been shown to be inactive in the digestive tract (Hochberg, Melnick and Oser, '45a).

The assay method is based upon the observation that under standardized conditions the urinary excretion of ascorbic acid parallels the quantity consumed (Melnick, Hochberg and Oser, '45). The test subjects of the preceding study were employed in the current investigation. They were healthy adult males, subsisting regularly on adequate, well-balanced dietaries. They excreted normal quantities of ascorbic acid before and after the administration of an oral test dose.

In the first availability study, comparison was made between the availability of ascorbic acid in raw fruits and vegetables (Control Period) and that derived solely from the fortified apple juice (Test Period). The ration consumed by the subjects during the Control Period was that routinely employed (Melnick, Hochberg and Oser, '45), modified only in that a greater amount of raw vegetables was included.<sup>5</sup> The values for the proximate constituents and vitamin content of this diet were the same as those described in the earlier paper (Melnick, Hochberg and Oser, '45).

The ascorbic acid-free basal ration, prior to supplementation with fortified apple juice, is described in table 2. In order to avoid the criticism directed at preceding studies, the diet was formulated free of all fruits and vegetables. The proximate and vitamin composition<sup>6</sup> of the unsupplemented diet was practically the same as that of the adequate basal ration except in ascorbic acid content. However, the addition of enriched apple juice, 460 ml in one-third portions at the end of each meal, contributed 115 mg of ascorbic acid, the same as that derived from the adequate diet containing raw fruits and vegetables.

During each period the subjects subsisted on the dietary for 2 weeks; the first 10 days constituted the adjustment period during which no urinary collections were made. Twenty-four-hour urine samples were collected during each of the next 4

<sup>5</sup> The ration included bread, butter, steak, milk, eggs, sugar, oranges, bananas, apples, lettuce, tomatoes, cucumbers, and a salad of raw cabbage, green beans, pepper and carrots.

<sup>6</sup> The methods employed for analyzing the rations were the same as those used in the preceding study (Melnick, Hochberg and Oser, '45).

days. On the final day, following the noon meal, a 200-mg test dose of extra ascorbic acid in aqueous solution was ingested. The precautions taken in collecting the urine samples to prevent loss of ascorbic acid have already been described (Melnick, Oser and Hochberg, '45). The photometric pro-

TABLE 2  
*Ascorbic acid-free diet.*

<i>Breakfast</i>			
2 slices of toast (wheat)	50 gm	1 cup of coffee	180 gm
2 butter squares	14 gm	(10 gm sugar)	
		(30 gm milk)	
<i>Dinner</i>			
1 steak (lean)	200 gm	1 bottle of beer	360 gm
3 slices of bread (rye)	97 gm	1 cup of coffee	190 gm
3 butter squares	21 gm	1 serving of cake	55 gm
<i>Supper</i>			
Noodle soup mix <sup>1</sup>	31 gm	3 butter squares	21 gm
2 fried eggs	81 gm	$\frac{1}{2}$ bottle of beer	180 gm
Cheddar cheese	70 gm	1 cup of coffee	180 gm
2 slices of bread (rye)	65 gm	1 serving of cake	55 gm
<i>Vitamin supplements<sup>2</sup></i>			
Thiamine	0.5 mg	Riboflavin	1.0 mg

*Analyses conducted on aliquots of the composite diet*

<i>Proximate analysis</i>	<i>Values found<sup>3</sup></i>	<i>Vitamin content</i>	<i>Values found<sup>3</sup></i>
Total weight	1840 gm	Ascorbic acid	0 mg
Total solids	521 gm	Thiamine	1.48 mg
Moisture	1319 gm	Thiamine: Calorie ratio	0.6
Protein	100 gm	Thiamine: Non-fat	
Fat (ether extract)	130 gm	calorie ratio	1.0
Ash	21 gm	Riboflavin	2.40 mg
Crude fiber	3 gm	Nicotinic acid <sup>4</sup>	19.8 mg
Carbohydrate (by difference)	267 gm		
Calorie value	2640 cal.		
Non-fat calories	1470 cal.		

<sup>1</sup> A mixture of glucose, monosodium glutamate, salt, spices, hydrogenated fat, chicken fat, and noodles.

<sup>2</sup> One-third aliquots taken at the end of each meal.

<sup>3</sup> Expressed in terms of total food consumed in the 3 meals.

<sup>4</sup> Predominantly as the amide.

cedure for estimating the vitamin content (Hochberg, Melnick and Oser, '43) included measurement of both dehydro- and reduced ascorbic acid. In this report only the total ascorbic acid values are presented, since in all cases approximately 90% of the vitamin in the urine was present as reduced ascorbic acid.

TABLE 1

*Urinary excretion of dietary and extra ascorbic acid by subjects ingesting raw fruits and vegetables as compared with fortified apple juice<sup>1</sup> as the sole source of dietary vitamin C.*

SUBJECT	DIET CONTAINING RAW FRUITS AND VEGETABLES				DIET CONTAINING FORTIFIED APPLE JUICE			
	Basal excretions			After 200 mg test dose of ascorbic acid	Basal excretions			After 200 mg test dose of ascorbic acid
	1st 24 hours	2nd 24 hours	3rd 24 hours		1st 24 hours	2nd 24 hours	3rd 24 hours	
	mg	mg	mg	mg/1st 24 hours	mg	mg	mg	mg/1st 24 hours
J. C.	36	45	57	111	48	41	42	139
E. M.	74	61	59	172	53	44	50	141
D. M.	40	44	43	163	41	46	37	96
M. H.	67	59	57	199	36	28	31	141
H. H.	22	23	27	103	36	52	39	121
Average	48	46	48	156	43	42	40	128

<sup>1</sup> Fortified from its original content of 0.7 mg to 30 mg of ascorbic acid per 120 ml. The fortified apple juice (460 ml per day) was taken in one-third portions at the end of each meal. The total intake of ascorbic acid was 115 mg per day, the same as that derived from the diet containing the raw fruits and vegetables.

In table 3 are listed the urinary excretion values obtained during the Control (raw fruit and vegetable) and Test (fortified apple juice) Periods. The differences in both the average basal and the test dose excretion values for the 2 periods were found not to be statistically significant ( $t = 0.8$  and  $1.5$ , respectively).<sup>7</sup> It may therefore be concluded that the ascorbic acid in the enriched apple juice is available to the same extent as the vitamin contributed by the raw fruits and vegetables in a natural, adequate diet.<sup>8</sup> These data, together with the

<sup>7</sup> A  $t$ -value of 2.3 would indicate that the difference between the averages of 2 sets of 5 observations is significant (95% probability).

<sup>8</sup> Considering that the average urinary excretion value for the 5 standardized subjects is reproducible to within  $\pm 10\%$  (Melnick, Hochberg and Oser, '45), it is apparent that the figures for comparative availability of the ascorbic acid in the fortified apple juice fall within the limits of precision of the biological assay.

results of the *in vitro* accelerated holding tests (see table 1), support the view of some nutritionists that apple juice is a favorable vehicle for supplying ascorbic acid to the human dietary.

The results of the assay presented do not answer the question whether or not the stabilizing factors occurring naturally in fruits and vegetables augment ascorbic acid availability. In both the control and test periods sufficient quantities of the unknown stabilizing agent may have been supplied to permit optimal absorption and utilization of the vitamin. That apple juice also contains factors which favor ascorbic acid stability has been demonstrated in tests previously described. To investigate the effect of the stabilizers, it is necessary that the ascorbic acid be taken during 1 of the periods in pure aqueous solution along with a basal ration free of all fruits and vegetables. Such a study was, therefore, also conducted.

In order to accentuate possible differences in urinary excretion values, the basal ration was supplemented with only 50 mg of ascorbic acid. This quantity in a natural diet is generally considered to be well above the minimum daily requirement (Federal Food, Drug and Cosmetic Act, '41). It is conceivable that when the ascorbic acid is taken in pure aqueous solution as a supplement to the ascorbic acid-free diet there might be negligible excretion of the vitamin in the urine, whereas administration of the vitamin in the apple juice containing stabilizing factors might be followed by appreciable excretion of the vitamin. If, in the absence of the stabilizing factors, excessive destruction of ascorbic acid occurs *in vivo*, depletion of tissue stores might result. This would be particularly evident from the excretion values obtained following dosage with the 200-mg test dose of extra ascorbic acid.

The time schedule of the second study was similar to that in the first. The same period was allowed for adjustment to the intake of 50 mg of ascorbic acid. Four consecutive urine samples were then collected. The first 3 were analyzed for estimation of the basal excretion value; the fourth for the de-

termination of the tissue stores of this vitamin as reflected by urinary excretion following postprandial ingestion of a 200-mg test dose of extra ascorbic acid. During the interval between the 2 urinary excretion studies, the subjects subsisted on a customary adequate diet furnishing approximately 115 mg of ascorbic acid per day. The urinary excretion values demonstrated that such an interval was ample to allow the subjects, on completion of the first portion of this study, to return to the state of nutrition they were in prior to the second phase.

TABLE 1

*Urinary excretion of dietary and extra ascorbic acid by subjects ingesting a vitamin C free diet supplemented with fortified apple juice<sup>1</sup> or with a pure aqueous solution of ascorbic acid.*

SUBJECT	DIET SUPPLEMENTED WITH FORTIFIED APPLE JUICE <sup>1</sup>				DIET SUPPLEMENTED WITH AQUEOUS ASCORBIC ACID SOLUTION <sup>2</sup>			
	Basal excretions			After 200 mg test dose of ascorbic acid	Basal excretions			After 200 mg test dose of ascorbic acid
	1st 24 hours	2nd 24 hours	3rd 24 hours		1st 24 hours	2nd 24 hours	3rd 24 hours	
	mg	mg	mg	mg/1st 24 hours	mg	mg	mg	mg/1st 24 hours
J. C.	15	13	8	32	14	17	13	52
E. M.	11	13	15	50	13	7	7	18
D. M.	12	12	12	24	18	16	12	46
M. H.	9	9	4	16	11	14	10	29
F. O.	11	15	8	47	16	16	15	58
Average	12	12	9	34	14	14	11	41

<sup>1</sup> Fortified from its original content of 0.7 mg to 30 mg of ascorbic acid per 120 ml.

<sup>2</sup> Both supplements furnished 50 mg of ascorbic acid per day.

In table 4 are listed the urinary excretion values obtained in the second availability study. Here also there was little difference in either the basal excretion figures or in the total values following dosage with extra ascorbic acid. No greater destruction occurs *in vivo* when the vitamin is taken in pure aqueous solution free of the stabilizing factors in fruits and vegetables. Indeed, calculations of the availability of ascorbic acid in the fortified apple juice gave values appreciably less than 100% when compared with the data obtained after dosage

with the aqueous vitamin solutions. The differences in both the average basal and the test dose excretion values for the 2 periods are, however, not statistically significant ( $t = 1.2$  and  $0.5$ , respectively).<sup>9</sup> It may therefore be concluded that the stabilizing factors in apple juice responsible for greater stability *in vitro* do not increase the availability of ascorbic acid to the organism. It would also follow that the enhanced biological potency of ascorbic acid in apple juice claimed by Crampton and Burton ('46) is not due to a factor which permits greater absorption and more effective utilization of the vitamin, but possibly to the use by these investigators of an assay procedure which is not specific for ascorbic acid alone but also estimates the activity of a covitamin (possibly citrin) required for the development of the odontoblast cells of the incisor teeth of guinea pigs.

Since in the first assay comparing the availability of ascorbic acid in fortified apple juice with that in a dietary containing raw fruits and vegetables, no significant differences in urinary excretion responses were observed, one may generalize by saying that the stabilizing factors in fruits and vegetables which protect ascorbic acid *in vitro* are ineffective, in the digestive system, in increasing ascorbic acid availability. Either the period for this effect to become apparent *in vivo* is too short or other protective factors may be present in the basal ration or in the digestive juices which mask the activity of the ascorbic acid stabilizing factors supplied by fruits and vegetables.

#### SUMMARY

Ascorbic acid is far more stable in fruit juices than in aqueous solutions. However, the stabilizing factors are ineffective in the digestive system in increasing ascorbic acid availability. On the basis of both *in vitro* and *in vivo* studies, it is concluded that apple juice is a favorable vehicle for ascorbic acid in the human dietary.

<sup>9</sup> It is to be expected that the reproducibility of the excretion responses would be poorer when the urinary ascorbic acid values are small.

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# VITAMIN A INTAKE IN CATTLE IN RELATION TO HEPATIC STORES AND BLOOD LEVELS<sup>1</sup>

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## ONE FIGURE

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The relation of vitamin A intake in animals to hepatic stores and blood levels has been reported in a number of papers. Lewis et al. ('42) determined hepatic stores and blood levels of vitamin A in rats after 6 weeks on a diet containing from 0 to 1000 I.U. of vitamin A daily. Vitamin A blood levels were found to reach a maximum with a daily intake of 100 I.U. Hepatic stores of vitamin A increased with increased intake throughout the range of vitamin A fed. Caldwell et al. ('45) studied hepatic stores of rats receiving 3, 6, and 12 I.U. of vitamin A per gm of dry ration during an experimental period of 300 days. The animals receiving 6 and 12 I.U. showed increasing hepatic stores of vitamin A throughout the experimental period. Braun ('45) studied hepatic stores and blood levels of carotenoids and vitamin A as affected by seasonal change and rations. Seasonal change in blood vitamin A levels was found to depend upon the carotene and vitamin A intake. Significant differences were found in hepatic stores of vitamin A in 3 of 4 dietary groups studied. Baumann et al. ('34) showed that 95% of the vitamin A stores in the rat are to be found in the liver, providing the body stores of vitamin A are adequate. Guilbert and Hart ('35),

<sup>1</sup> Colorado Agricultural Experiment Station, Scientific Series Article no. 249.

working with cattle, found 93% of the body stores of vitamin A in the liver. The principal stores of carotene were found in the body fat. Frey and Jensen ('46) showed that a rapid decrease in hepatic stores of vitamin A and carotene occurred in cattle on a fattening ration consistent with good feeding practice. Riggs ('40) has shown that night blindness will develop in cattle in from 46 to 266 days on a ration practically devoid of carotene. The time required to produce night blindness was found to depend upon age, the nature of the ration, and the ration fed previously.

In the present paper data are presented on hepatic stores and blood serum levels of vitamin A and carotene in cattle on a carotene-free basal ration containing varying amounts of vitamin A in the form of high potency fish oil. The paper represents in part an investigation of the cause of liver abscess formation in cattle. The experiment was based on data derived from 2 previous experiments (Frey and Jensen, '46; Jensen et al., '47).

#### EXPERIMENTAL DESIGN AND RESULTS

One hundred and fourteen Hereford steers of about 8 months of age were taken from native grass pasture and divided into 6 lots of 19 animals each. Lot 1 was slaughtered at the beginning of the experiment to determine the initial stores of vitamin A and carotene in the blood and liver. The remaining 5 lots were placed on experimental rations December 7, 1945. Table 1 gives the basal ration and vitamin A supplement for each lot. The rations were hand fed in amounts demanded by the animals for maximum consumption.

The vitamin A supplement was prepared by mixing a high potency oil, guaranteed to contain not less than 200,000 I.U. of vitamin A per gm, with a low potency oil to give a mixture containing approximately 25,000 I.U. per gm. The vitamin A content of the mixture was determined colorimetrically by means of the Carr-Price reaction (Carr and Price, '26) using an Aminco type-F photoelectric colorimeter. Crystalline vitamin A alcohol was used as the reference standard. One  $\mu$ g

of vitamin A alcohol was considered equivalent to 4.3 I.U. of vitamin A. The vitamin A-containing oil was mixed well with the rolled barley, this in turn being mixed with the other ration constituents in the feed trough. The animals were weighed at 28-day intervals and the vitamin A supplement adjusted to give the intake per pound weight of animal as given in table 1.

TABLE 1

*Vitamin A supplements, average daily rations, weight gains, and grade of carcass.*

LOT NO.	2	3	4	5	6
I.U. vitamin A/lb. body wt. daily	0	25	100	200	500
Average daily rations (lb.)					
Rolled barley	4.56	5.58	5.55	5.58	5.58
Dried beet pulp	4.47	5.47	5.44	5.47	5.47
Wet beet pulp	4.88	5.42	5.44	5.41	5.39
Ground oat straw	2.43	2.62	2.62	2.66	2.65
Cottonseed meal	0.94	0.99	0.99	0.99	0.99
Mineral	0.03	0.03	0.05	0.05	0.04
Salt	0.04	0.05	0.05	0.07	0.06
Average initial weight (lb.)	460	461	460	461	460
Average weight—166 days (lb.)	760	846	834	846	849
Average weight—277 days (lb.)	842	999	993	1000	1025
Average daily gain (lb.)	1.52	2.07	2.03	2.07	2.14
Government grade of carcass					
Choice	6	11	9	12	11
Good	9	8	10	7	8
Commercial	3	0	0	0	0
Condemned	1	0	0	0	0

Blood samples were taken from each lot of animals at 27 and 83 days. Nine animals from each lot were slaughtered after 166 days of the experiment and the remaining 10 at 280 days. Liver tissue was obtained from each animal at the time of slaughter. Blood samples were taken from the animals at the last weighing previous to slaughter, corresponding to 159 and 277 days, respectively. The sampling technique and methods of analysis for vitamin A and carotene in liver tissue were the same as described by Frey and Jensen ('46). Serum vitamin A and carotene were determined by the method of

Kimble ('39). The ration constituents contained no carotene or preformed vitamin A.

#### DISCUSSION OF RESULTS

In table 1 are given the average daily consumption of ration constituents and the weight gains in pounds for each lot during the experimental period of 277 days. The animals in lot 2 show an average daily gain of 1.52 pounds as compared to an average of 2.08 pounds for the other 4 lots. The carcasses in lot 2 showed less finish and graded lower than did those in the other lots. The 4 lots receiving vitamin A supplement graded about equally, indicating that an intake of 25 I.U. of vitamin A per pound body weight daily is sufficient to maintain finish.

Indications of vitamin A deficiency were observed in the animals in lot 2 during the fourth month of the feeding period. Typical eye involvement with impaired vision, as manifested by some change in the ability to discern objects in daylight, was observed in 8 of the 10 animals in lot 2 at 277 days.

The data in table 2 give the average values found for serum levels and hepatic stores of vitamin A and carotene for each lot while on the experimental rations. The serum levels of carotene decreased rapidly. At the 83-day period and thereafter no trace of carotene was found in the serum. This is in contrast to the hepatic stores where definite amounts of carotene were found at 280 days. At the end of 27 days the carotene content of the serum had dropped to approximately  $\frac{1}{2}$  the initial value, there being no significant difference between lots. In lot 2 the serum values of vitamin A reached a maximum at 27 days. Subsequent values at 159 and 277 days showed a rapid decrease of vitamin A. Lot 3, which received 25 I.U. of vitamin A per pound body weight daily, showed increasing serum values up to 83 days. Between 83 and 159 days the serum levels of vitamin A started decreasing. Lot 4, which received 100 I.U., showed increasing serum levels of vitamin A for 159 days. Lot 5, which received 200 I.U., and lot 6, which received 500 I.U. showed similar patterns. Each

TABLE 2

Serum levels and hepatic stores of vitamin A and carotene expressed as micrograms of constituent per 100 ml of serum or per gm of liver.<sup>1,2</sup>

DAYS ON RATION	LOT 2			LOT 3			LOT 4			LOT 5			LOT 6		
	$\bar{x}$	$\sigma$	$\eta$	$\bar{x}$	$\sigma$	$\eta$	$\bar{x}$	$\sigma$	$\eta$	$\bar{x}$	$\sigma$	$\eta$	$\bar{x}$	$\sigma$	$\eta$
<i>Serum vitamin A</i>															
0 <sup>3</sup>	30.0	7.1	17												
27	34.4	8.2	13	34.7	5.7	11	39.0	6.8	14	42.0	6.8	17	38.0	9.5	14
83	34.4	12.1	9	43.5	15.5	10	39.4	7.0	9	42.6	11.1	10	41.6	11.1	11
159	13.7	2.1	9	40.2	4.0	9	52.7	4.6	9	50.4	7.8	9	53.7	8.1	9
277	5.2	2.3	10	29.2	6.0	10	48.6	5.1	10	55.1	8.5	10	57.2	8.8	10
<i>Serum carotene</i>															
0 <sup>3</sup>	44.5	12.5	17												
27	20.4	18.7	13	23.3	11.6	11	21.6	7.2	14	23.4	13.3	17	25.1	11.2	14
<i>Hepatic vitamin A</i>															
0 <sup>3</sup>	47.1	22.7	16												
166	0.4	0.3	9	2.6	1.5	9	18.4	4.8	9	52.6	25.3	9	127.0	32.2	9
280	0.2	0.1	10	1.4	0.8	10	17.4	7.1	10	40.0	10.4	10	130.0	50.9	10
<i>Hepatic carotene</i>															
0 <sup>3</sup>	3.6	2.3	16												
166	0.68	0.33	9	0.65	0.19	9	0.56	0.12	9	0.70	0.26	9	0.48	0.17	9
280	0.35	0.28	10	0.29	0.12	10	0.29	0.14	10	0.22	0.07	10	0.23	0.19	10

<sup>1</sup>  $\bar{x}$  = Arithmetic mean of values found;  $\sigma$  = standard deviation;  $\eta$  = number of animals from which tissue samples were obtained.

<sup>2</sup> See table 1 for vitamin A supplement given each lot.

<sup>3</sup> Serum levels and hepatic stores at 0 days the same for all lots.

showed increasing levels of serum vitamin A throughout the experimental period of 277 days. An intake of 200 I.U. or more of vitamin A per pound body weight daily was sufficient to meet requirements in addition to increasing serum levels of vitamin A. Under these experimental conditions an intake of between 100 I.U. and 200 I.U. of vitamin A per pound body weight daily maintained a nearly maximum serum level.

The increasing pattern of serum vitamin A in lots 2, 3 and 4 up to 27, 83 and 159 days, respectively, is difficult to explain. The animals had been shipped from the Texas Panhandle and placed on native grass pasture about 3 weeks prior to being placed on experimental rations. It is probable that the animals were in the process of building up hepatic stores of carotene and serum vitamin A at the beginning of the experiment. One would expect a time factor to be involved in establishing blood levels of vitamin A from hepatic stores of carotene when the latter are increasing. Since these conditions most likely prevailed it is possible that the relatively large hepatic reserves of carotene were responsible for the increasing serum levels of vitamin A that were observed.

At the end of 166 days the average hepatic stores of carotene had dropped from 3.6  $\mu\text{g}$  per gm of liver to 0.61  $\mu\text{g}$ . At the end of 280 days the average carotene content was 0.28  $\mu\text{g}$  per gm of liver. As previously shown by Frey and Jensen ('46), the rate of depletion of hepatic stores of vitamin A and carotene diminishes with decreasing hepatic stores of either of the 2 constituents. Hickman ('46) postulated the existence of a half-time recovery or depletion period for body stores of a vitamin. On the basis of such a theory the greatest loss of carotene stores would be predicted to occur during the initial period of the experiment. The small difference in the hepatic reserves of carotene for the different lots at 166 and 280 days indicates that vitamin A does not exert an appreciable sparing action on hepatic stores of carotene.

The vitamin A content of the livers of the animals in lot 2 was found to be 0.2  $\mu\text{g}$  per gm of liver after 280 days on a carotene-free ration. The significance of such a low value is

questionable when one considers the influence of interfering substances in the Carr-Price reaction. The animals receiving 200 I.U. or less of vitamin A per pound body weight daily showed decreased hepatic stores of vitamin A at the end of the experimental period of 280 days. Those receiving 500 I.U. showed increased hepatic stores at the end of the experimental period. No significant increase in hepatic stores of vitamin A was found between 166 and 280 days.

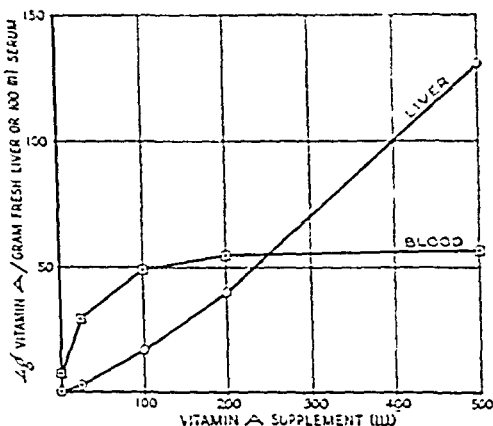


Fig. 1. Hepatic stores at 280 days, and blood serum levels at 277 days, of vitamin A compared with varying vitamin A supplements in I.U. per pound body weight daily.

In figure 1 hepatic stores of vitamin A at 280 days and serum levels of vitamin A at 277 days are compared with varying vitamin A supplements (tables 1 and 2). It will be observed that hepatic stores of vitamin A increase in practically a linear relationship with vitamin A intake. The serum levels of vitamin A show a rapid increase up to an intake of 100 I.U. of vitamin A per pound body weight daily. Above an intake of 200 I.U. the rate of increase in serum vitamin A is small. This suggests that an intake of 100 I.U. of vitamin A per pound body weight daily is sufficient to maintain nearly maximum serum levels in cattle under the conditions of the experiment. This is supported by reference to hepatic stores of vitamin A as given in table 2. Hepatic stores of vitamin A

began increasing above the initial values between an intake of 200 I.U. and 500 I.U. of vitamin A per pound body weight daily when the initial reserves were as given in table 2.

Figure 1 shows no simple relationship to exist between serum levels and hepatic stores of vitamin A. This suggests that the body mechanism controlling serum levels of vitamin A is different from that controlling hepatic stores.

Table 3 summarizes the results obtained in making a comparison of individual values between lots to determine the

TABLE 3

*Orthogonal comparison of lots to determine the significance of blood and liver values of vitamin A as given in figure 1.<sup>1</sup>*

LOTS COMPARED	$\sigma$	$\sigma\bar{x}$	M.S.D.	
			0.05	0.01
Blood — 277 days				
2 and 3	4.17	1.87	3.90	5.34
3 and 4	2.00	0.90	1.87	2.55
4 and 5	8.84	3.95	8.27	11.30
5 and 6	41.00	18.40	38.50	52.60
Liver — 280 days				
2 and 3	0.51	0.23	0.48	0.66
3 and 4	5.10	2.28	4.78	6.53
4 and 5	10.30	4.60	9.62	13.20
5 and 6	32.50	14.52	30.40	41.60

<sup>1</sup>  $\sigma$  = Standard deviation;  $\sigma\bar{x}$  = standard error; M.S.D. = minimum significant difference between means at the 5 and 1% levels.

significance of the average values shown in figure 1. The values for the minimum significant differences between means show that for blood levels of vitamin A the first 2 orthogonal comparisons differ very significantly, while the last 2 do not differ significantly. This indicates that blood levels of vitamin A, under the conditions of the experiment, reach a maximum value at an intake of between 100 I.U. and 200 I.U. of vitamin A per pound body weight daily. For the liver values of vitamin A all 4 orthogonal comparisons show highly significant differences. This indicates that hepatic stores of vitamin A increase



with increasing intake of vitamin A throughout the range fed in the experiment.

#### SUMMARY AND CONCLUSIONS

Hereford steers of about 8 months of age were maintained on a carotene-free basal ration containing vitamin A supplements at levels of 0, 25, 100, 200 and 500 I.U. per pound body weight daily. Serum levels of vitamin A and carotene were determined at 0, 27, 83, 159 and 277 days, and hepatic stores at 0, 166 and 280 days.

The animals receiving only the basal ration showed a lower ration consumption and lower average daily gain than those receiving vitamin A supplement. The ration consumption and weight gains were about the same for all lots receiving vitamin A supplement.

Dietary vitamin A did not exert a sparing action on hepatic stores of carotene. Blood stores of carotene were depleted sooner than were hepatic stores. Hepatic stores of vitamin A increased in practically a linear relationship with intake throughout the range of vitamin A supplement fed. Serum levels increased rapidly up to an intake of 100 I.U. of vitamin A per pound body weight daily. An intake of 100 I.U. of vitamin A per pound body weight daily will maintain nearly maximum serum levels of vitamin A in cattle under the conditions of the experiment.

Serum levels and hepatic stores of vitamin A appear to be controlled by different body mechanisms.

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# STUDIES OF THYROID TOXICITY

## I. A NUTRITIONAL FACTOR WHICH ALLEVIATES THE TOXICITY OF INGESTED THYROID SUBSTANCE<sup>1</sup>

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The importance of nutrition in experimental hyperthyroidism and its effect on the course of the syndrome have been emphasized by many investigators. The vitamin requirements of hyperthyroid animals appear to be increased. Abelin ('30) found that increasing the casein content of a thyrotoxic diet decreased the severity of the symptoms. The casein used was a crude preparation and may have carried other essential nutrients with it. There is a great deal of evidence which indicates an increased requirement for vitamin A (Abelin et al., '30; Abelin, '33; von Euler and Klussman, '32; Logaras and Drummond, '38; Sure and Buchanan, '37b) and in guinea pigs for ascorbic acid (Svirbely, '35; Sure and Theis, '39) during experimental hyperthyroidism.

Most attention has been focused on the vitamins of the B complex. These vitamins, where their function has been elucidated, have been found to be associated in the enzyme systems which yield energy to the organism. The thyroid gland through its hormone appears to exert a regulatory influence on these energy-yielding metabolic processes. At present, the specific function of the thyroid hormone is not clear.

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It is quite logical to assume that an increased metabolic rate which results from the administration of a thyrotoxic material may necessitate an increased dietary intake of members of the B complex. The work of many workers confirms such a hypothesis (Abelin, '30; Cowgill and Palmieri, '33; Himwich et al., '31, '32). The partial protective effect exerted by vitamin B<sub>1</sub> was first shown by Sure and Smith ('34), Sure and Buchanan ('37a) and confirmed by Drill and Sherwood ('38). Thyrotoxicity has been shown to be accompanied by a reduction of vitamin B<sub>1</sub> content of the tissues (Drill, '37) and also of their cocarboxylase activity (Peters and Rossiter, '39). Vitamin B<sub>1</sub> alone did not give as adequate protection as was obtained when yeast or a yeast concentrate was added to the ration (Drill, '37; Sure and Smith, '34; Sure and Buchanan, '37a).

As more members of the B complex became available in pure form, their effects in counteracting thyrotoxicity were studied. Drill and Overman ('42) found that pyridoxine and calcium pantothenate were required in larger quantities during experimentally induced thyrotoxicosis. Their ration contained a minimal quantity of yeast. Abelin ('45, '46), feeding a crude ration, observed a beneficial effect when massive doses of calcium pantothenate were administered to hyperthyroid rats. Ershoff and Hershberg ('45) were unable to alleviate the symptoms resulting from thyroid administration by greatly increasing the calcium pantothenate level of the ration. Katzenelbogen, Alexrod and Elvehjem ('41) observed that increasing the nicotinic acid content of the ration did not affect the gross symptoms of thyrotoxicity, but did prevent the accompanying decrease in the coenzyme I content of the tissues.

Ershoff and Hershberg ('45) found that rats fed a purified ration very high in the members of the vitamin B complex known to be required by the rat grew poorly when desiccated thyroid was added. The feeding of a yeast-containing ration resulted in much better growth and survival. This indicated that some factor present in yeast, other than the vitamins

added, exerted an antithyrototoxic effect. Their purified ration did not contain inositol, biotin, nicotinic acid, folic acid, and p-aminobenzoic acid.

The present investigation was undertaken to determine the nature of the growth stimulating, antithyrototoxic agent in yeast and other sources. It was designed to show whether this effect was due to one of the known members of the vitamin B complex or whether an as yet unknown factor was responsible.

### PROCEDURE

Male, weanling albino rats of the Sprague-Dawley strain and weighing 35-45 gm were used in this study. It has been shown by earlier work (Drill, '38a, b) that male rats are more sensitive to administered thyroid substance than are females.

TABLE 1  
*Composition of the rations.*

	RATION A	RATION B
	%	%
Casein (hot alcohol extracted)	22	22
Sucrose	63.5	56.5
Salts IV (Phillips and Hart, '35)	4.5	4.5
Corn oil	5	5
Dried brewer's yeast		12

To each kg of the above rations the following crystalline vitamins were added: thiamine hydrochloride 3 mg, riboflavin 6 mg, nicotinic acid 20 mg, pyridoxine hydrochloride 3 mg, calcium pantothenate 30 mg, choline chloride 2 gm, p-aminobenzoic acid 50 mg, biotin 0.1 mg, folic acid 0.25 mg, and inositol 1 gm. Each animal received weekly 3 drops of Abbott Haliver oil U.S.P. (Haliver oil contains 60,000 U.S.P. units of vitamin A and 600 U.S.P. units of vitamin D per gm.) The above rations contain all the known members of the vitamin B complex, added in crystalline form.

Each animal was housed in an individual mesh-bottomed cage and was weighed weekly. All animals were given food and water *ad libitum*.

The composition of the rations used is given in table 1. Supplements were added to the basal as per cent of the basal, unless otherwise indicated. In all experiments both

negative and positive controls were included. The negative control received the purified ration plus desiccated thyroid, while the positive control received the purified ration alone.

After preliminary orienting experiments, it was found that a 4-week growth period could be used as an assay of the antithyrototoxic properties of a material. This assay was used in these studies.

TABLE 2

*Effect on growth and survival of graded amounts of ingested thyroid substance.*

LOT NO. <sup>1</sup>	RATION	THYROID CONTENT	AVERAGE SURVIVAL	NO. ALIVE AFTER 84 DAYS	AVERAGE WEIGHT AT		
					4 weeks	8 weeks	12 weeks
		% added <sup>2</sup>	days <sup>3</sup>		gm	gm	gm
13	A	0.0	84	3	181 ± 19	275 ± 21	302 ± 33
14	A	0.1	81	2	140 ± 18	195 ± 17	172 ± 16(2)
15	A	0.25	38	0	98 ± 8		
16	A	0.5	35	0	72 ± 3(2) <sup>4</sup>		
17	B	0.0	84	3	162 ± 27	256 ± 48	300 ± 60
18	B	0.1	84	3	154 ± 21	198 ± 8	207 ± 15
19	B	0.25	71	2	129 ± 11	179 ± 3(2)	186 ± 7(2)
20	B	0.5	39	0	109 ± 16		

<sup>1</sup> Three rats per lot.

<sup>2</sup> The desiccated thyroid preparation used in the above experiment was Parke-Davis Desiccated Thyroid gland. It was 50% stronger than U.S.P. and contained 0.3% iodine in organic combination.

<sup>3</sup> The experiment was terminated after 84 days. Where any of the animals survived the full length of the experimental period, 84 days was averaged into the survival to obtain the *average survival*.

<sup>4</sup> Numbers in parentheses indicate the number of animals still alive at that time.

## RESULTS

In preliminary experiments it was found that the levels of desiccated thyroid fed by Ershoff and Hershberg ('45) were too high for the strain of rats used in these studies. This may also have been due to sex differences. On dietary levels of 0.5 and 1.0% desiccated thyroid, no protective effect of yeast could be demonstrated.

Table 2 summarizes the experiment performed to determine the optimal level of desiccated thyroid at which changes in growth and survival due to yeast administration would be

most marked. Desiccated thyroid was fed at levels of 0.0, 0.1, 0.25, and 0.5% added to the purified (ration A) and yeast (ration B) rations. The data (table 2) indicate that there was a graded response to the various levels of thyroid fed. This especially can be seen for the growth depression and is also reflected in the survival data. A comparison of lots 13-16 with lots 17-20 clearly shows that yeast exerted a marked antithyrotoxic effect. The differences in growth and survival resulting from yeast feeding were most marked on a level of 0.25% desiccated thyroid. Therefore this level was used in all subsequent experiments. It can also be seen that a ration (A), containing all the known B vitamins at levels which were much higher than adequate for normal animals, was incapable of supporting a good rate of growth when supplemented with thyroid.

#### *Other possible antithyrotoxic materials*

The efficacy of a number of other materials in exerting an antithyrotoxic effect was determined. Since there is no agreement in the literature (Drill and Overman, '42; Abelin, '45, '46; Ershoff and Hershberg, '45) concerning the effect of pantothenic acid on thyrotoxicosis, the action of very large quantities of this vitamin was determined. Two groups of rats (table 3, experiment A) were fed the purified ration and a low and high level, respectively, of calcium pantothenate. The animals receiving 330 mg of the vitamin per kg of ration showed no significant improvement in growth or survival when compared to those receiving 30 mg per kg of ration. It may be concluded that an *augmented* intake of calcium pantothenate exerted no beneficial effect in these experiments.

Alopecia has been shown to be a symptom of inositol deficiency in mice (Woolley, '41) and rats (Cunha et al., '43). The reported occurrence of alopecia during the course of thyrotoxicosis (Ershoff and Hershberg, '45) indicated a possible increased requirement of inositol for rats receiving desiccated thyroid. Lots 22 and 24 (table 3, experiment A)

demonstrated that the addition of 2% inositol to the thyroid-containing ration exerted no antithyrototoxic effect.

In attempts to find other sources of the antithyrototoxic factor(s), dried whole liver powder,<sup>2</sup> yeast nucleic acid, and 1:20 liver powder,<sup>2</sup> were fed. A comparison of lots 22 and 25 shows that the dried liver preparation fed at 5% of the ration is an excellent source of the antithyrototoxic factor(s); an average weight difference of 62 gm was noted at the end of 4 weeks. The beneficial effect of liver was exhibited throughout an 11-week period while all of the rats in lot 22 died after 6 to 8 weeks.

Dried whole liver, when added at a 5% level, exerted a greater beneficial effect than did 12% dried brewer's yeast. This marked protection, by dried whole liver powder, was reflected in both growth and survival of the animals. Dried whole liver powder was also found to be active when fed at a 3% level (experiments C and D, table 3).

The addition of 5 gm of liver powder 1:20<sup>2</sup> (roughly equivalent to 30 gm of dried whole liver powder) per kg of ration had a marked protective effect as is clearly shown by a comparison of lots 38 and 40. The effect of yeast nucleic acid was determined since this substance occurs in high concentration in dried yeast. It can be seen from the data in table 3 (experiment B) that the addition of 0.5% yeast nucleic acid had no beneficial effect.

A comparison of lot 28 with lot 35 indicates that yeast still exerted some antithyrototoxic action when added to the ration at a level of 5%.

#### *Stability of the antithyrototoxic activity of liver*

In order to determine the stability of the antithyrototoxic material(s) in liver, dried whole liver powder was subjected to the rather drastic treatments described below. The dry powder was suspended in 2-3 times its weight of distilled water and then divided into 3 portions and treated as follows:

<sup>2</sup> Wilson.



One sample was autoclaved for 1 hour at 15 pounds. The other 2 were adjusted to pH 1.0 and 10.0 with hydrochloric acid and sodium hydroxide, respectively, and then autoclaved for 1 hour at 15 pounds. These were then neutralized with sodium hydroxide and hydrochloric acid. Each of the 3 preparations was dried and ground to a fine powder before use.

The treated preparations were fed at levels equivalent to 5% whole liver powder in 1 experiment (B) and at a 3% level in another (D). The data in table 3 indicate that, at either of the levels fed in these studies, none of the above treatments resulted in a loss of the antithyrotoxic activity of the whole liver powder.

In a subsequent experiment an extract of dried whole liver powder which had antithyrotoxic activity was heated at 100°C. for 12 hours with no loss of activity. It can be concluded from the above experiments that the activity of dried whole liver powder as an antithyrotoxic agent is not destroyed by heating in water, acid, or alkaline solutions.

#### DISCUSSION

The data presented clearly indicate that there is present in liver a factor(s) which is essential for the *growth* of rats suffering from experimentally induced hyperthyroidism. When the ration contained all known members of the vitamin B complex, at levels much higher than accepted adequate levels, thyrotoxicity was severe; also, further addition of calcium pantothenate and inositol gave no protection, whereas the various liver preparations exerted antithyrotoxic effects. In other experiments, too detailed to describe in this paper, doubling the vitamin levels described in table 1 had no noticeable effect on growth and survival.

It may well be that thyroid feeding *increases the requirement* of the growing animal for some as yet unknown factor(s) which the animal ordinarily obtains in adequate quantities through tissue or intestinal synthesis. Another possibility which must be considered is that thyroid feeding results in *decreased synthesis* of some factor(s) essential for growth.

TABLE 3  
Sources and stability of the antithyrotoxic factor(s).

LOT NO. <sup>1</sup>	DATION	THYROID SUBSTANCE <sup>2</sup>	SUPPLEMENT	AVERAGE WEIGHT 3 WEEKS
		% added		gm
<i>Experiment A</i>				
21	A	0.0	None	154 ± 10
22	A	0.25	None	81 ± 16
23	A	0.25	300 mg calcium pantothenate per kg of ration	65 ± 15
24	A	0.25	2% mesitol	78 ± 16
25	A	0.25	5% dried whole liver substance <sup>3</sup>	143 ± 11
26	B	0.25	None	124 ± 13
<i>Experiment B</i>				
27a	A	0.0	None	159 ± 2
27b	B	0.0	None	177 ± 6
28	A	0.25	None	103 ± 1
29	A	0.0	5% dried whole liver powder	195 ± 9
30	A	0.25	5% dried whole liver powder	139 ± 15
31	A	0.25	5% autoclaved whole liver powder	159 ± 6
32	A	0.25	5% whole liver powder autoclaved in acid solution	147 ± 15
33	A	0.25	5% whole liver powder autoclaved in alkaline solution	147 ± 11
34	B	0.25	None	141 ± 10
35	A	0.25	5% dried brewer's yeast	135 ± 13
36	A	0.25	0.5% yeast nucleic acid	94 ± 12
<i>Experiment C</i>				
37	A	0.0	None	139 ± 9
38	A	0.38	None	119 ± 4
39a	A	0.0	3% dried whole liver powder	190 ± 10
39	A	0.38	3% dried whole liver powder	141 ± 11
40	A	0.38	5 gm liver powder 1:20 per kg of ration <sup>4</sup>	142 ± 10
<i>Experiment D</i>				
52	A	0.0	None	179 ± 14
53	A	0.38	None	129 ± 3
59	A	0.38	3% whole liver powder autoclaved in alkaline solution	163 ± 8
60	A	0.38	3% dried whole liver powder	142 ± 13

<sup>1</sup> Four rats per lot.

<sup>2</sup> The desiccated thyroid used in experiments A and B of the table was a Parke Davis (0.3% iodine) preparation, in C and D, Wilson desiccated thyroid USP (0.2% iodine) was used. The substitution was made on an equi iodine basis.

<sup>3</sup> Wilson.

<sup>4</sup> Liver powder 1:20 (Wilson) is prepared by water extraction of whole liver. One gm is equivalent to 20 gm of fresh liver.

Still a third alternative is that liver and yeast contain a substance which directly antagonizes the action of the thyroid hormone. Further work is necessary to determine how the beneficial effect of liver in counteracting thyrotoxicity is exerted.

The antithyrotoxic factor(s), as can be seen from the data, is not destroyed by heating in neutral, acid, or alkaline suspension. Its presence in liver powder 1:20<sup>3</sup> indicates it to be water soluble. The factor(s) reported in this paper does not appear to be identical with the monkey anti-anemia factor (also present in liver) reported recently (Ruegamer et al., '47) since the latter factor is heat labile.

While this paper was in preparation, Ershoff ('47) reported the presence of a factor in liver, other than the known B vitamins, which was necessary for the growth of immature rats fed desiccated thyroid. The results reported in this paper are in agreement with his data. Work is now in progress on the fractionation of liver preparations in order to determine the properties of, and to purify the active material observed in these studies. Progress which has been made in that direction will be reported in a later paper.

#### SUMMARY

1. The presence in yeast of an antithyrotoxic factor, which has been previously reported, has been confirmed in these studies.
2. Liver has been found to be a better source of the antithyrotoxic material than yeast. This factor is not one of the known B vitamins. It is heat stable and under the experimental conditions employed is not destroyed by autoclaving in acid or alkaline solution.
3. Calcium pantothenate and inositol did not exert an antithyrotoxic effect when fed at very high levels in a purified ration.

<sup>3</sup> Wilson.

TABLE 1  
Sources and stability of the antithyrototoxic factor(s).

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		% added		gm
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25	A	0.25	5% dried whole liver substance <sup>3</sup>	143 ± 11
26	B	0.25	None	124 ± 13
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27 <sup>a</sup>	A	0.0	None	159 ± 2
27b	B	0.0	None	177 ± 6
28	A	0.25	None	103 ± 1
29	A	0.0	5% dried whole liver powder	195 ± 9
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<sup>3</sup> Wilson.

<sup>4</sup> Liver powder 1:20 (Wilson) is prepared by water extraction of whole liver. One gm is equivalent to 20 gm of fresh liver.

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# THE EFFECT OF THE INGESTION OF HIGH LEVELS OF RIBOFLAVIN ON THE AMOUNT IN THE MILK AND URINE<sup>1</sup>

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During the course of studies on the effect of the ingestion of synthetic thiamine, riboflavin, nicotinic acid and pantothenic acid on the amounts of these vitamins in the milk of the cow it was observed that by the microbiological method the riboflavin content of the milk from cows fed the synthetic vitamins showed varying degrees of increase from none up to a maximum of a 2-fold increase. Usually the milk showed a very distinct greenish-yellow color, characteristic of aqueous solutions of riboflavin. Samples of milk from cows fed rather large amounts of riboflavin, when measured by the standard fluorometric procedure, gave values approximately 5 times higher than the values obtained microbiologically.

This observation suggested that at least in certain species the apparent riboflavin value of the milk following the ingestion of riboflavin might differ by as much as 500%. This posed a problem of both applied importance and possibly one of comparative biochemistry of a species difference in metabolism of riboflavin. Furthermore it seemed important to determine by feeding experiments with rats which of the 2 assay

<sup>1</sup>The authors are indebted to Merek and Company for the riboflavin, to J. A. Gray for providing the sheep used in these investigations, and to Patricia G. Sparks and Helen Keene for technical assistance.





diluted urine or filtrate from the milk with potassium permanganate and hydrogen peroxide (Scott et al., '46). The values obtained by this treatment are referred to in the tables under the column headed " $\text{KMnO}_4$ ." This fraction was then adsorbed on florisil and eluted with a 20% pyridine-2% acetic acid solution. The values obtained by this treatment are referred to in the tables under the column headed " $\text{KMnO}_4$  plus florisil."

## RESULTS AND DISCUSSION

*Milk*

The data on the riboflavin values for milk obtained by the different analytical procedures are presented in table 1. Since the goat and sheep responded in a similar manner to the

TABLE 1

*Apparent riboflavin in goat's and ewe's milk. (Values in  $\mu\text{g}$  per ml.)*

ANIMAL <sup>1</sup>	DIETARY TREATMENT	MICRO-BIOLOGICAL	FLUOROMETRIC		
			Untreated	$\text{KMnO}_4$	$\text{KMnO}_4$ plus florisil
Goat no. 1	Basal	3.7	4.0	3.8	3.9
Goat no. 1	Riboflavin	4.3	19.8	20.0	19.8
Goat no. 2	Basal	3.4	3.7	3.7	3.6
Goat no. 2	Riboflavin	3.5	25.7	25.2	24.0
Goat no. 3	Basal	2.4	2.6	2.5	2.5
Goat no. 3	Riboflavin	3.7	14.7	14.2	12.0
Sheep no. 1	Basal	5.7	6.9	6.3	5.3
Sheep no. 1	Riboflavin	6.7	44.8	45.0	34.2
Sheep no. 2	Basal	5.4	6.3	5.6	5.2
Sheep no. 2	Riboflavin	7.8	37.6	36.6	26.2

<sup>1</sup> The weights of the animals starting with goat no. 1 and proceeding down the table were 50.0 kg, 43.6 kg, 47.7 kg, 54.5 kg and 50.9 kg.

ingestion of riboflavin and have similar values for the milk, the data for the 2 species are considered together.

The fluorometric values on the untreated normal milk average about 15% higher than the microbiological values. This indicates that goat's and ewe's milk contain some non-ribo-

procedures measured the true biological activity of the riboflavin in the milk.

### EXPERIMENTAL

In these studies milch goats and sheep were used since metabolism cages were available permitting the quantitative collection of the urine simultaneously with the collection of the milk. With the goats the full amount of milk secreted was withdrawn from the udder, but with ewes it was not possible to obtain all of the milk. There was also a marked difference in the amount of milk produced by the goats, and for these reasons the data are more uniform and consistent when expressed on a ml basis rather than on a 24-hour basis.

The urine and milk were collected for 2 consecutive days. Two gm of synthetic riboflavin were then administered in a gelatin capsule for each of 3 consecutive days. The urine and milk were collected on the second and third day of ingestion of the riboflavin. Precautions were taken to prevent destruction of the riboflavin in the milk and urine by photolysis.

The urine and milk collected both before and after the ingestion of riboflavin were assayed by the standard microbiological method of Snell and Strong ('39). Riboflavin was also measured by the fluorometric procedure with a Coleman photofluorometer after 3 different treatments of the samples. The milk was autoclaved with 0.1 N HCl for 30 minutes. After adjusting the pH to about 4.6 the aliquot was made up to volume and filtered. The urine was merely adjusted to the proper pH and diluted. A sufficiently large sample was prepared so that all assays could be made from the initial preparation.

The milk and urine samples for the microbiological assays received no treatment other than that previously mentioned. The riboflavin was also measured fluorometrically for the milk and urine samples so prepared without any attempt to remove non-riboflavin fluorescent compounds. This value is referred to in the tables as "untreated" under fluorometric. The second fluorometric procedure consisted in treating the

diluted urine or filtrate from the milk with potassium permanganate and hydrogen peroxide (Scott et al., '46). The values obtained by this treatment are referred to in the tables under the column headed " $\text{KMnO}_4$ ." This fraction was then adsorbed on florisil and eluted with a 20% pyridine-2% acetic acid solution. The values obtained by this treatment are referred to in the tables under the column headed " $\text{KMnO}_4$  plus florisil."

## RESULTS AND DISCUSSION

*Milk*

The data on the riboflavin values for milk obtained by the different analytical procedures are presented in table 1. Since the goat and sheep responded in a similar manner to the

TABLE 1

*Apparent riboflavin in goat's and ewe's milk. (Values in  $\mu\text{g}$  per ml.)*

ANIMAL <sup>1</sup>	DIETARY TREATMENT	MICRO-BIOLOGICAL	FLUOROMETRIC		
			Untreated	$\text{KMnO}_4$	$\text{KMnO}_4$ plus florisil
Goat no. 1	Basal	3.7	4.0	3.8	3.9
Goat no. 1	Riboflavin	4.3	19.8	20.0	19.8
Goat no. 2	Basal	3.4	3.7	3.7	3.6
Goat no. 2	Riboflavin	3.5	25.7	25.2	24.0
Goat no. 3	Basal	2.4	2.6	2.5	2.5
Goat no. 3	Riboflavin	3.7	14.7	14.3	12.0
Sheep no. 1	Basal	5.7	6.9	6.3	5.3
Sheep no. 1	Riboflavin	6.7	44.8	45.0	34.2
Sheep no. 2	Basal	5.4	6.3	5.6	5.2
Sheep no. 2	Riboflavin	7.8	37.6	36.6	26.2

<sup>1</sup> The weights of the animals starting with goat no. 1 and proceeding down the table were 50.0 kg, 43.6 kg, 47.7 kg, 54.5 kg and 50.9 kg.

ingestion of riboflavin and have similar values for the milk, the data for the 2 species are considered together.

The fluorometric values on the untreated normal milk average about 15% higher than the microbiological values. This indicates that goat's and ewe's milk contain some non-ribo-

flavin fluorescent compounds. Treating with potassium permanganate and hydrogen peroxide gave values slightly lower than the untreated values. The microbiological values agreed very well with the values obtained after treating with  $\text{KMnO}_4$  and adsorbing on florisol. The average riboflavin content of the 5 samples of normal milk by the microbiological method was  $4.12 \mu\text{g}$  per ml as compared to  $4.10 \mu\text{g}$  per ml by the fluorometric method following treatment with  $\text{KMnO}_4$  and adsorbing on florisol.

Following the ingestion of 2 gm of riboflavin daily there was an increase of only 26% in the riboflavin content of the milk as measured by the microbiological method. The average riboflavin content of the milk from the animals on the basal diet was  $4.12 \mu\text{g}$  per ml as compared to  $5.20 \mu\text{g}$  per ml following the ingestion of riboflavin. The magnitude of the increase is very small considering the amount of riboflavin ingested. These observations differ from the findings with women where there appears to be a more direct correlation between the intake of riboflavin and the concentration in the milk (Neuweiler, '37).

Following the ingestion of 2 gm daily of synthetic riboflavin there was a marked increase in the riboflavin value of the milk of all animals as measured fluorometrically. Using the " $\text{KMnO}_4$  plus florisol" procedure the riboflavin values were 5 to 6 times that obtained by the microbiological procedure. Obviously 1 of these methods represents an error probably due to fluorescent compounds measured by the fluorometric procedure which do not have biological activity. The validity of this assumption was tested by feeding experiments with rats.

### *Rat growth*

Milk from 2 goats and 1 ewe was fed to groups of rats and the growth response used as a measure of the true riboflavin present. Milk collected during the period the basal diet was fed and during the period of riboflavin ingestion was evaporated on purified diets at levels to provide the same amount

of riboflavin in the diets when measured by the microbiological procedure. The diet used for measuring the biological activity of the riboflavin in the milk was the same as that previously described (Schweigert and Pearson, '47) except for the riboflavin. On the basis of the fluorometric value the diet containing the riboflavin milk provided 150  $\mu$ g or more of riboflavin per 100 gm of diet. This is adequate to permit maximum rates of gain by the rat. That the diet was adequate in all factors

TABLE 2

*Growth of rats supplemented with milk as source of riboflavin.*

SOURCE OF RIBOFLAVIN	$\mu$ G RIBOFLAVIN/100 GM DIET		BODY WEIGHT <sup>1</sup>		
	Micro- biological	KMnO <sub>4</sub> plus florisil	Initial	Final	Gain per week
		<i>Goat's milk</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
Normal milk					
7 ml/100 gm diet	23.4	25.3	34.6	52.0	5.8
Riboflavin milk					
7 ml/100 gm diet	24.1	179.2	39.0	53.5	4.8
Synthetic riboflavin	150.0	150.0	41.2	103.0	20.6
		<i>Ewe's milk</i>			
Normal milk					
5.1 ml/100 gm diet	29.3	27.1	42.3	60.6	6.1
Riboflavin milk					
4.4 ml/100 gm diet	29.3	150.0	42.8	59.1	5.4
Synthetic riboflavin	150.0	150.0	42.2	89.5	15.8

<sup>1</sup> Data are averages. There were 6 rats in each group and the length of the feeding period was 3 weeks.

except riboflavin is apparent from the good gains made by the rats fed the diet supplemented with 150  $\mu$ g of synthetic riboflavin per 100 gm of diet. The results for the milk from the 2 goats were in essential agreement. Therefore, to conserve space the growth data for milk from only goat no. 2 and ewe no. 1 are presented in table 2.

The rats fed the milk from the goat on the basal diet and the milk collected during the period of riboflavin ingestion

made essentially the same rate of gain. The average weekly gain of the rats receiving the diet containing the milk collected during the basal period was 5.8 gm while the rats fed the diet containing the riboflavin milk gained an average of 4.8 gm per week. The diets containing the ewe's milk each provided 29.3  $\mu$ g riboflavin per 100 gm according to the microbiological assay. By the fluorometric method the diet containing the riboflavin milk should have provided 179.2  $\mu$ g of riboflavin per 100 gm. The average weekly gains for the 2 groups of rats were 6.1 and 5.4 gm, respectively, for the rats receiving the diet containing the normal milk and the riboflavin milk. From these data it is clear that the riboflavin measured fluorometrically above that measured microbiologically is not active for the rat.

It is of interest that the rats receiving the milk collected during the period of riboflavin feeding made slightly lower gains than the rats receiving the diets containing the milk from the animals on the basal diet. The average weekly gain by the rats fed the normal milk from the second goat (not shown in table 2) was 6.4 gm as compared with 4.8 gm by the rats fed the diet containing the milk collected during the period of riboflavin feeding. While the data here are meager, the possibility that metabolites formed from riboflavin by the goat and sheep may have a depressing effect on growth of rats should not be overlooked.

### *Urine*

The ingestion of riboflavin resulted in an increase of around 3-fold as measured microbiologically in the level of riboflavin in the urine of the goats. The increase was of a smaller order for the ewes. The volume of urine voided by ewe no. 1 during the period of riboflavin ingestion was 940 ml per 24 hours as compared to 730 ml during the basal period. The daily excretion of riboflavin by this animal was greater during the period of riboflavin feeding than during the basal period, but it does not show up on a ml basis due to the difference in volume of urine.

The microbiological values on the urines collected during the basal period are in good agreement with the values obtained by the fluorometric procedure after treating with  $\text{KMnO}_4$  and adsorbing on florisol.

On the basis of microbiological assay the maximum recovery in the urine of the 2 gm ingested was 4.8 mg. The average recovery in the urine of ingested riboflavin was 4.0 mg, 4.8 and 2.9 mg, respectively, for goats 1, 2 and 3. The corresponding recoveries for the ewes were 1.0 and 2.8 mg for numbers

TABLE 3

*Apparent riboflavin in the urine of sheep and goats. (Values in  $\mu\text{g}$  per ml.)*

ANIMAL <sup>1</sup>	DIETARY TREATMENT	MICRO-BIOLOGICAL	FLUOROMETRIC		
			Untreated	$\text{KMnO}_4$	$\text{KMnO}_4$ , plus florisol
Goat no. 1	Basal	4.3	5.1	5.0	4.6
Goat no. 1	Riboflavin	15.3	156.0	160.0	107.0
Goat no. 2	Basal	2.9	5.0	5.0	3.6
Goat no. 2	Riboflavin	7.8	164.0	143.8	124.0
Goat no. 3	Basal	4.0	6.1	6.1	4.5
Goat no. 3	Riboflavin	12.6	147.0	135.6	136.0
Sheep no. 1	Basal	7.2	7.0	6.9	6.8
Sheep no. 1	Riboflavin	6.7	138.4	124.0	110.0
Sheep no. 2	Basal	2.8	3.7	3.7	2.7
Sheep no. 2	Riboflavin	5.6	98.0	87.0	73.0

<sup>1</sup> The weights of the animals starting with goat no. 1 and proceeding down the table were 50.0 kg, 43.6 kg, 47.7 kg, 54.5 kg and 50.9 kg.

1 and 2. These data suggest that the riboflavin is rapidly metabolized by the sheep and goat to compounds without biological activity.

Following the ingestion of riboflavin the values obtained by the fluorometric procedure following treatment with  $\text{KMnO}_4$  and adsorbing on florisol were from 7 to 16 times the values obtained by the microbiological assay. Adsorbing on florisol following treatment with  $\text{KMnO}_4$  was more effective in removing non-riboflavin fluorescent pigments than the  $\text{KMnO}_4$  treatment only. From these data it is apparent that

when goats and sheep are fed large amounts of riboflavin, compounds having fluorescent properties but not biological activity are formed. The non-riboflavin fluorescent compound is secreted into the milk and excreted by the renal pathway.

The fluorescent compound or compounds without biological activity that are formed by the goat and sheep from ingested riboflavin have not been identified. It is possible that the ribose moiety of the riboflavin molecule is broken off and that 6:7 dimethyl-alloxazine or lumichrome is produced. This compound is biologically inactive, but it is highly fluorescent. Whether the site of formation of the biologically inactive compound is in the rumen or in the tissues following absorption remains to be determined.

#### *Effect of ingestion of large amounts of riboflavin by rats*

From a comparative standpoint it was of interest to determine whether or not the rat when given large amounts of riboflavin would excrete non-riboflavin fluorescent compounds similar to the goat and sheep.

For these studies 3 mature rats were used for each level of riboflavin administered. The urine was collected for 2 consecutive days. Ten mg of riboflavin were administered by stomach tube on each of 3 consecutive days, and the urine collected on the second and third days. The urines were assayed for riboflavin by the microbiological method and by each of the 3 fluorometric procedures. Of the latter only the value obtained by treating with  $\text{KMnO}_4$  and adsorbing on florisol will be referred to here. The 10 mg of riboflavin on the basis of body weight, was approximately equivalent to the level ingested by the goats and sheep.

On the basal diet the average daily urinary excretion of riboflavin as measured by the microbiological procedure was 15.9  $\mu\text{g}$  as compared with 14.7  $\mu\text{g}$  by the fluorometric procedure. Following the ingestion of 10 mg of riboflavin the average daily urinary excretion measured by the microbiological procedure was 114.3  $\mu\text{g}$  as compared to a value of 137.0  $\mu\text{g}$  by the



fluorometric method. To another group of 3 rats 20 mg of riboflavin were administered daily. This is about double the amount ingested by the sheep and goats per kg of body weight. On the basal diet the average daily riboflavin excretion was 16.3  $\mu$ g and 19.4  $\mu$ g as measured by the microbiological and fluorometric methods, respectively. The corresponding figures during the period of riboflavin ingestion were 208.4 and 223.4  $\mu$ g of urinary riboflavin. The magnitude of the differences between the biological values and the fluorometric values is essentially the same for the urine from the rats on the basal diets as for the rats receiving the riboflavin. From these data it is apparent that the sheep and goat differ from the rat in their metabolism of riboflavin in that the rat does not form appreciable amounts of biologically inactive compounds that are measured by the fluorometric procedures for riboflavin.

### *Observations on human beings*

The question whether man responds in a manner similar to the rat or the goat and sheep on the ingestion of relatively large amounts of riboflavin is of considerable interest. Should human beings metabolize ingested riboflavin in a manner similar to the goat and sheep, data on excretion studies may differ considerably depending on whether the analytical data were obtained by the microbiological method or fluorometric method. On the other hand, if man metabolizes ingested synthetic riboflavin in a manner similar to the rat, it would make no essential difference whether the riboflavin determinations were made by the fluorometric method or by the microbiological method.

For these studies 2 male adults were used. The urine was collected quantitatively for 2 days. Twenty mg of riboflavin in the form of tablets were ingested daily for the 3 succeeding days. The urine was collected on the second and third days of the period. The level of riboflavin intake was about 10 times the daily allowance recommended by the Food and Nutrition Board ('45).

There was no significant difference in the riboflavin values obtained by the microbiological assay and the fluorometric method where the urine was treated with  $\text{KMnO}_4$  and adsorbed on florisil. Following the ingestion of riboflavin the urine of subject no. 1 contained  $6.28 \mu\text{g}$  of riboflavin per ml as measured microbiologically and  $6.47 \mu\text{g}$  by the fluorometric method. The corresponding values for the urine of subject no. 2 were  $4.50 \mu\text{g}$  and  $3.37 \mu\text{g}$  per ml by the microbiological and fluorometric procedures, respectively.

From these data it is apparent that at the levels of riboflavin that would normally be ingested by human beings, that comparable results for riboflavin in the urine could be expected using either the microbiological or standard fluorometric procedures.

#### SUMMARY

The oral administration of large amounts of riboflavin to goats and sheep increased the true riboflavin content of the milk by only about 26%. Measured by the fluorometric procedure the riboflavin values were for the milk approximately 5 times greater than the values obtained by the microbiological method and for the urine the fluorometric values were about 12 times higher. The riboflavin content of the milk as measured by the microbiological assay agreed well with the growth response of rats fed the milk as a source of riboflavin.

The microbiological and fluorometric values for the riboflavin content of the urine of rats were in good agreement even when massive doses of 10 or 20 mg of riboflavin were administered daily. The microbiological and fluorometric values were in good agreement for urine from humans that had been fed 20 mg of riboflavin per day.

Goats and sheep fed large amounts of riboflavin evidently form a fluorescent compound that is measured by the fluorometric procedure for riboflavin. The unidentified compound secreted into the milk and excreted by the renal pathway is not biologically active.

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# SOME STUDIES ON THE NUTRITIVE VALUE OF BUTTER FATTY ACIDS<sup>1</sup>

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The effect of volatile fatty acids on the weight gain of young rats was compared with that of other fatty acid fractions of butter fat by Schantz et al. ('40) and Brown and Bloor ('45). While both investigations indicate a dietary inferiority of the short-chain fatty acids, their effect as part of butter fat is still doubtful. Schantz et al. fed them mixed with corn oil and liquid skim milk, a diet which admittedly produces diarrhea (Hart, '45), whereas Brown and Bloor gave them as part of a Sherman B diet. The latter workers also observed a laxative action and reduced the fatty acid content of the volatile fatty acid diet from the 10% level of the control diet to 6%. In the presence of lactose the saturated fatty acids of butter were found to be superior to the unsaturated fatty acids by Schantz et al., but not by Brown and Bloor. Considering the work of Henry et al. ('45), Henderson et al. ('45), Jack et al. ('45), and particularly the recent discovery of the growth-promoting effect of vaccenic ( $\Delta^{11,12}$ —octadecenoic) acid in summer butter (Boer et al., '47), one would expect the unsaturated rather than the saturated fatty acid fraction of butter to be more effective in promoting the weight gain of young rats. The effect of

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unsaturation as such is by no means clearly established, since the fatty acid composition of butter underwent some alteration in the feeding experiments thus far reported. The amount and concentration of vitamin A stored in the livers of rats given raw carrots has also been reported to vary with the nature of the dietary fatty acids (Brown and Bloor, '45). On an average, rats fed unsaturated fatty acids stored more and higher concentrations of vitamin A than those on a saturated fatty acid diet. Whether this effect operates on the conversion of carotene to vitamin A can be elucidated by feeding preformed vitamin A instead of carotene.

#### EXPERIMENT 1

Three groups of rats were given, respectively, butter fatty acids, their non-volatile portion, and hydrogenated butter fatty acids. The fatty acids of butter were prepared as follows: 5 pounds of butter, bought on the local market, were melted on the steam-bath and then allowed to solidify in a beaker in the ice-chest. The aqueous phase was drained off after puncturing the fat cake. The butter fat was washed with water in the molten state and again separated from water as before. This was repeated once more. Saponification was carried out by refluxing the fat on a steam-bath for 2 hours with 8 liters of 10% KOH in 95% methanol in a round bottom flask. At the end of this period the flask was converted into a continuous liquid extractor of the type described by Hilditch ('40). The extraction of the non-saponifiable matter with low-boiling petroleum ether was continued until the soaps gave a negative Carr-Price test. This took about 10 hours. The soap solution was then transferred to a 5-gallon bottle, cooled by adding chopped ice, and acidified to Congo Red paper with HCl. Ether was added, the bottle stoppered and laid on its side. To mix its contents the bottle was rolled back and forth, then put in an upright position and allowed to stand until the ether separated out. The ether solution of fatty acids was syphoned off into another 5-gallon bottle. After repeated extraction of the fatty acids with ether, the com-

bined ether extracts were washed with a small amount of water to remove most of the alcohol, then dried overnight with calcium chloride. The solvent was removed from the fatty acids by vacuum distillation. This resulted in 1220 gm of fatty acids with an average molecular weight (determined by titration) of 251.0 and an iodine value (Wijs' method) of 59.1. The product was divided into 3 portions.

A 400-gm portion of butter fatty acids was steam distilled for  $5\frac{1}{2}$  hours, at which time about 5 liters of distillate had collected in the receiver. The residue was allowed to solidify in the ice-box. The cake of fatty acids was separated from the water phase and dissolved in petroleum ether. To this solution a petroleum ether extract of the water phase was added. After drying and evaporation as before, 361 gm of non-volatile fatty acids remained. Their average molecular weight was 264.4.

Another 400-gm portion of the original fatty acids was introduced into a high pressure hydrogenation bomb in the dry, molten state, together with about 5 gm Raney nickel catalyst. The hydrogenation was carried out in the absence of solvents at 2200 lbs. and  $150^{\circ}\text{C}$ . with agitation for 2 hours. To remove the catalyst the hydrogenated fatty acids were dissolved in petroleum ether and shaken with fairly concentrated hydrochloric acid. The petroleum ether solution was then washed with water, dried, and evaporated. The iodine value of the hydrogenated product was 40.6.

The diets used were similar to those employed by Brown and Bloor ('45). The Sherman B diet was modified in such a way that the fatty acids under investigation constituted the fat component to the extent of 9.9% of the diet. Three rations were prepared containing, respectively, the original butter fatty acids, the non-volatile fatty acids, and the partially hydrogenated fatty acids. Diets were prepared in 1 kg batches by kneading the molten fatty acids into the mixture of whole wheat flour, skim milk powder, and salt. Rats were fed as much as they would consume within a 2-day period, the remainder of the food being stored in the ice-box.

Thirty albino rats of the Wistar strain, 15 male and 15 female, 23-24 days old, were distributed as equally as possible with respect to sex, age, litter, and weight over the 3 experimental groups. Although no special precautions were taken to prevent rats from spilling their food, the use of heavy, deep cups, only half full of diet, permitted a good rough estimate of the relative weekly food consumption per group of males and females, because the amount of food spilled was small and about equal. The rats were weighed weekly and their gains recorded.

Desynon,<sup>3</sup> a solution containing 500 U.S.P. units vitamin A and 50 U.S.P. units vitamin D<sub>3</sub> in 90% alcohol per drop, was used as a source of vitamins A and D. The solution was diluted 5 times with milk. Each rat received a drop of this preparation, i.e., 100 units vitamin A and 10 units vitamin D on the day the experiment was started and every week thereafter, except on the last day of the experiment. Solutions of Desynon in milk were prepared immediately before use and fed by means of the special dropper supplied with the product. Due to gradual deterioration the vitamin A content of Desynon fell to such an extent that the animals received only about half of the initial dose by the end of the experiment. At the end of the fifth week the rats were killed by illuminating gas, and their livers examined for vitamin A by the methods of McCoord and Luce-Clausen ('34) and Clausen et al. ('42). No appreciable amounts of vitamin A could be detected.

Table 1 summarizes the results of the first experiment. The mean weight gains since the start of the experiment together with their standard errors, the weekly food consumption, and the efficiency of food conversion, expressed as the percentage by weight of food converted into body substance are given for each week. The effect of volatile fatty acids on weight gain was evaluated by calculating Fisher's "t" for the difference between the mean weight gains of male and female rats on the diet of the original and of the non-volatile fatty acids.

<sup>3</sup> Winthrop Chemical Co., Inc.



This gave the following "t" values for the 5 consecutive weeks of the experiment: Males: 1.1, 1.1, 2.0, 1.5, 1.4; Females: 0.6, 0.5, 0.3, 1.4, 1.6. The corresponding probability values, taken from Fisher's table (Fisher, '38) are: Males: 0.3, 0.3, 0.1-0.05, 0.2-0.1, 0.2-0.1; Females: 0.6-0.5, 0.6, 0.8-0.7, 0.2-0.1. Regarding any probability level below the conventional 0.05 as significant, it is evident that there is no significant difference between the weight gain of males or females

TABLE 1

*Weight gains, food consumption, and efficiency of food conversion of rats on diets containing various butter fatty acids.*

	WEEK	NON-VOLATILE FATTY ACIDS		ORIGINAL FATTY ACIDS		PARTLY HYDROGENATED FATTY ACIDS	
		♂	♀	♂	♀	♂	♀
Cumulative mean weight gains in gm	1	22 ± 3	18 ± 2	24 ± 3	17 ± 3	11 ± 2	17 ± 2
	2	46 ± 3	39 ± 3	48 ± 2	37 ± 7	37 ± 6	39 ± 7
	3	70 ± 4	59 ± 7	75 ± 3	57 ± 10	54 ± 10	57 ± 5
	4	94 ± 2	75 ± 7	98 ± 5	67 ± 8	69 ± 17	73 ± 4
	5	20 ± 3	89 ± 8	124 ± 5	80 ± 8	88 ± 26	89 ± 2
Mean weekly food con- sumption per rat, gm	1	45	45	51	44	44	48
	2	62	61	66	56	60	58
	3	70	64	72	62	61	63
	4	75	68	74	60	62	63
	5	82	71	84	60	68	63
Mean effi- ciency of food con- version	1	49	40	47	39	25	35
	2	39	34	36	36	43	38
	3	34	31	38	32	28	29
	4	32	24	31	17	24	25
	5	32	20	31	22	28	25

fed the original butter fatty acids and those fed the non-volatile portion of these acids.

The effect of hydrogenation of the dietary fatty acids on weight gain was similarly evaluated by calculation of Fisher's "t" for the difference between the mean weight gains of males and females on the original and on the hydrogenated fatty acid diet. The following "t" values were obtained: Males: 7.8, 3.5, 4.1, 3.2, 2.8; Females: 0, 0.4, 0, 1.3, 2.1. The correspond-

ing probability values are: Males:  $< 0.01$ ,  $< 0.01$ ,  $< 0.01$ ,  $0.02-0.01$ ,  $0.02$ ; Females:  $> 0.9$ ,  $> 0.9$ ,  $> 0.9$ ,  $0.3-0.2$ ,  $0.1-0.05$ . This means that the weight gain of males, but not of females, is significantly lower on a diet containing partially hydrogenated fatty acids than on the original butter fatty acid diet.

The figures on food consumption and conversion indicate that the efficiency usually falls off with time, and that it is usually lower for females than for males of the same group. Some, though inconsistent, differences appear between the males fed partially hydrogenated and the original butter fatty acids. Hydrogenation seems to have reduced the food utilization of the males, sometimes even below that of the females of the same group.

#### EXPERIMENT 2

In order to determine the effect of complete hydrogenation of the dietary butter fatty acids on weight gain and vitamin A storage a similar experiment was set up with completely hydrogenated fatty acids and larger vitamin supplements. The method of preparation and purification of the fatty acids of butter was the same as that described under experiment 1. About half of the fatty acids were hydrogenated at 1950 lbs. and  $150^{\circ}\text{C}$ . in the presence of about 7 gm Raney nickel and 100 ml absolute alcohol with agitation for 8 hours. This time the iodine value of the fatty acids dropped to 1.1 after hydrogenation. Two diets were prepared, 1 containing the original fatty acids of butter, the other their hydrogenated counterpart. Twenty Wistar rats, 10 male and 10 female, all 29 days old, were evenly distributed over the 2 groups. The Desynon solution was analyzed weekly for vitamin A by evaporating an aliquot portion of a drop in a colorimeter tube, dissolving it in purified (McCoord and Luce-Clausen, '34) chloroform, and then following the procedure of Clausen et al. ('42). Instead of milk, glycerol was used to reduce the strength of the alcohol in Desynon. Mixtures of 2 parts of glycerol and 1 part of Desynon were given by dropper in such amounts that the rats received about 300 units vitamin A at the start

of the experiment and 1 week thereafter, 400 units at the end of the second week, 500 units at the end of the third week, and 600 units at the end of the fourth week. The number of units of vitamin D given was about one-tenth that of vitamin A. All rats were killed at the end of the fifth week and their livers analyzed for vitamin A by the method previously mentioned.

TABLE 2

*Weight gains, food consumption, and efficiency of food conversion of rats on diets containing unchanged and completely hydrogenated butter fatty acids.*

	WEEK	ORIGINAL		COMPLETELY HYDROGENATED	
		♂	♀	♂	♀
Cumulative mean weight gains, in gm	1	20 ± 4	14 ± 2	21 ± 4	16 ± 2
	2	46 ± 7	34 ± 5	46 ± 4	37 ± 4
	3	73 ± 12	53 ± 6	67 ± 4	53 ± 5
	4	98 ± 16	67 ± 2	91 ± 7	69 ± 4
	5	121 ± 21	75 ± 4	110 ± 7	77 ± 5
Mean weekly food consumption, in gm	1	55	52	64	54
	2	71	61	72	66
	3	86	67	82	71
	4	88	70	83	74
	5	100	69	87	74
Mean efficiency of food conversion	1	36	27	33	30
	2	37	33	35	32
	3	31	28	26	23
	4	28	20	29	22
	5	23	12	22	11

The results of the second experiment are summarized in tables 2 and 3. Statistical evaluation of the weight differences between rats fed the original and the completely hydrogenated butter fatty acids by Fisher's "t" test gave for males: 0.4, 0, 1.0, 0.8, 1.0, and for females: 1.4, 1.0, 0, 0.8, 0.7. Corresponding P values are, for males: 0.7, > 0.9, 0.4-0.3, 0.5-0.4, 0.4-0.3, and for females: 0.2, 0.4-0.3, > 0.9, 0.5-0.4, 0.5. Thus, there was no significant difference between the weight gain of rats receiving the original butter fatty acids and those

fed the completely hydrogenated fatty acids. The efficiency of food conversion can again be seen to fall off with time. It is smaller for females than for males, but there is no appreciable difference between the 2 groups in that respect.

Table 3 gives the mean vitamin A concentrations in I.U./100 gm of wet liver, the mean amount of vitamin A stored in the livers of each of the 2 groups of male and female rats used in experiment 2, and the standard errors of the means. Evaluation of the difference between mean vitamin A concentrations of the 2 groups by Fisher's "t" test gave 0.9 for males, and 0.8 for females. This corresponds to probability values of

TABLE 3  
*Vitamin A in the livers of rats on diets containing unchanged,  
and completely hydrogenated butter fatty acids.*

	SEX	MEAN CONCENTRATION IN I.U./100 GM WET TISSUE	MEAN AMOUNT IN I.U.
Original	M	2201 $\pm$ 390	224 $\pm$ 20
	F	5823 $\pm$ 1310	403 $\pm$ 94
Completely hydrogenated	M	1858 $\pm$ 653	176 $\pm$ 55
	F	6567 $\pm$ 1290	505 $\pm$ 105

0.4 and 0.5-0.4 for males and females, respectively. Differences between mean amounts yielded "t" values of 1.6 and 1.5, and P values of 0.2-0.1 and 0.2-0.1 for males and females in the order given. Thus, there was no significant difference between the amounts or concentrations of vitamin A in the livers of either male or female rats, regardless of whether the fatty acids of butter were fed in their original or completely hydrogenated form. Females of both groups stored about twice as much vitamin A as males of both groups.

#### DISCUSSION

If the increased weight gain is the criterion chosen as a measure of nutritive value, the *ad libitum* method of feeding should be of greater practical value than the paired feeding

technique (Boutwell et al., '41). Food found superior by the *ad libitum* method might owe its growth-promoting effect to greater palatability or stimulation of the appetite or some other incidental properties, but in any case increased growth response will indicate a superiority in some practically relevant respect.

Steam distillation strips the butter fatty acids of those of low molecular weight, as shown by an increase in the molecular weight. By comparing rat growth on a fatty acid supplement lacking the volatile fatty acids with that on an equal quantity of the untreated fatty acids of butter the effect of the volatile fraction can be determined. The chief advantages of this method may be stated as follows: (1) No reduction in fat intake (Brown and Bloor, '45) is necessary, since such a regimen does not produce diarrhea. (2) Other fats need not be added to equalize the fat intake without increasing the proportion of volatile fatty acids (Schantz et al., '40). (3) The volatile fatty acids are diluted by the specific amount and proportion of the other naturally occurring fatty acids, increasing the practical significance of the results. The results indicate that the volatile fatty acids, as they naturally occur in butter fat, have no particular influence on growth or efficiency of food utilization. The harmful effect of these fatty acids, observed by Schantz et al. and by Brown and Bloor, must be ascribed to the absence of the other butter fatty acids from their diets. On the other hand, the more flavorful short-chain fatty acids did not appreciably increase appetite and food consumption of weanling rats. Hilditch and Jasper-son ('42) found 2 changes to occur in the fatty acid composition of butter when the diet of milch cows is changed from hay to fresh grass, namely, reduction of the percentage of volatile fatty acids, and an increase in the amount of oleic acid. The growth-promoting effect of summer butter now appears to be due not to the former change, as has been suggested by them, but to the increase in the oleic acid fraction, which also contains vaccenic acid.

It seems difficult to reconcile the postulate of a growth-promoting saturated fatty acid with the finding that the unsaturated fatty acid vaccenic acid has growth-promoting activity. An attempt was made to test both contentions by feeding the hydrogenated fatty acids of butter and comparing their influence on growth with that of untreated butter fatty acids. In such an experiment unsaturation is the only variable — the composition of the fatty acid mixture remains unchanged. In the first experiment on the effect of hydrogenation on the growth-promoting value of the fatty acids of butter the iodine value of the fatty acids was lowered from 59.1 to 40.6. The growth of male rats on the partially hydrogenated fatty acid diet was definitely inferior to that of their litter-mates on untreated fatty acids, and their efficiency in converting food into body substance was somewhat reduced. In the second experiment, however, when the fatty acids were for all practical purposes completely hydrogenated, there was no difference in weight gain or in the efficiency of food conversion between animals of both sexes on hydrogenated and untreated fatty acids. Since rats grew as well on completely hydrogenated fatty acids as on the original fatty acids of an iodine value of about 60, unsaturation clearly could not have been the sole factor in determining the growth rate in experiment 1. Except for the iodine value of the hydrogenated fatty acids the diet of the animals in experiment 1 differed from that used in experiment 2 in 1 important respect. In experiment 1 each rat received weekly from 50 to 100 units of vitamin A and about 10 units of vitamin D. In the course of the whole experiment a rat could not have received more than 500 units of vitamin A and 50 units vitamin D. But the total amount of vitamin A consumed per rat in the second experiment was around 2000 units, together with about 200 units vitamin D. It is conceivable that the reduction of the unsaturated growth-factor of Boer et al. ('47), namely vaccenic acid, or of unsaturated fatty acids in general by partial hydrogenation had a deleterious effect on rat growth by lowering the efficiency of food conversion only

when the vitamin intake was relatively low, but not when a more generous vitamin supplement was given. Boer and Jansen ('42) have also observed that upon addition of vitamin D (but not A) to winter butter the difference in response between summer and winter butter disappears, while addition of vitamin D to summer butter is without effect. By the same token partial hydrogenation in experiment 1 had a greater effect when the vitamin supply in the diet was relatively scanty, than complete hydrogenation in experiment 2 where higher doses of vitamin D offset the removal of unsaturated material.

It is interesting to note that Boer and Jansen ('42) as well as Brown and Bloor ('45) used only male rats. Female rats may have the ability to synthesize unsaturated fatty acids more efficiently than males, or else they may use their supply of fat-soluble vitamins more economically, as evidenced by the greater storage of vitamin A in the livers of females. Sex differences in the metabolism of lipids or lipid-soluble vitamins are not at all unusual.

Hydrogenation of butter fatty acids has either no effect on their growth-promoting value or else it reduces it. In the absence of other fats hydrogenation does not improve growth, even in the presence of relatively large amounts of lactose from the skim milk powder in the Sherman B diet. It seems unlikely therefore that butter owes its growth-promoting effect to a saturated fatty acid.

Desynon was chosen as a source of vitamins A and D so that each rat could be given an identical, approximately known vitamin supplement without using a carrier oil which might influence the absorption of fat-soluble vitamins. The fatty acids used were free of vitamin A and carotene. The stored vitamin of all livers was therefore derived from an equal amount of preformed dietary vitamin A, and any difference in storage should be expected to be due to differences in vitamin A metabolism. As there was no significant difference between the amount or concentration of vitamin A stored in the livers of animals receiving either butter fatty

acids or their completely hydrogenated counterpart, it is not considered likely that unsaturated fatty acids in general or a specific unsaturated fatty acid present in butter fat have a specific influence on the absorption and storage of vitamin A. This does not preclude the possibility that they may in some way effect the utilization of carotene.

#### SUMMARY

1. There was no statistically significant difference in weight gain or efficiency of food conversion during a 5-week period between 2 groups of 10 young rats (5 males and 5 females) each, regardless of whether the fat component of a Sherman B diet was made up of the fatty acids of butter or the same fatty acids, stripped of their low molecular weight fraction by steam-distillation.

2. In a similar experiment a significant difference in weight gain and some difference in the efficiency of food conversion was observed when the effect of butter fatty acids as part of the Sherman B diet was compared with that of the partially hydrogenated fatty acids. Weight gain and efficiency of food utilization were decreased only in the male rats of the group fed the hydrogenated fatty acids, and only when the intake of vitamins A and D was relatively low.

3. The amount and concentration of vitamin A stored in the livers of rats fed preformed vitamin A was not significantly affected by complete hydrogenation of the butter fatty acids in the diet.

#### ACKNOWLEDGMENTS

The author gratefully acknowledges help and advice given him during the course of this work by Dr. W. R. Bloor and Dr. A. B. McCoord.

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## MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1948 Award of \$1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the Spring of 1948.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1 to December 31 the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1947 must be in the hands of the Nominating Committee by January 10, 1948. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

E. N. TODHUNTER  
*University of Alabama*  
*University, Alabama*

CHAIRMAN, MEAD JOHNSON NOMINATING COMMITTEE



# SELF SELECTION OF DIET

## VI. THE NATURE OF APPETITES FOR B VITAMINS <sup>1</sup>

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ONE FIGURE

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It was shown in a previous paper that appetites for diets containing thiamine, riboflavin, or pyridoxine were developed by rats previously fed diets deficient, respectively, in these vitamins, while normal animals showed no such preferences (Scott and Quint, '46b). It was concluded that appetites for these vitamins were related to nutritional need for them, and thus that they were either learned appetites or true hungers. As one of the principal differences between learned appetites and true hungers it was postulated that learned appetites for nutritional essentials require the animal to have previous experience of the effects of the beneficial diet, in order to learn to associate such effects with the diet, while true hungers are always coexistent with nutritional need and should require no learning process. Harris et al. ('33) concluded that the appetite for thiamine was learned as a result of beneficial experience. This appeared probable since rats which had previously been on a thiamine-deficient diet and had then formed the habit of eating a food of a particular flavor containing thiamine, showed no appetite for thiamine in food of a different flavor.

The present report outlines a method of determining whether an appetite such as that observed in these researches

<sup>1</sup> Contribution no. 639 from the Department of Chemistry, University of Pittsburgh. Aided by grants of the Nutrition Foundation, Inc., and the Buhl Foundation.

is learned, i.e., is a behavior conditioned by previous experiences, or represents a true hunger. The method was applied to the appetites for thiamine, riboflavin, and pyridoxine, with the result of showing their essential similarity as learned appetites.

#### EXPERIMENTAL

To decide whether the vitamin appetites were learned or whether they were true hungers, advantage was taken of the fact that establishment of a learned appetite for a vitamin may conceivably result in an association of beneficial effect of the vitamin with some recognizable physical characteristic of the diet, while a true vitamin hunger should be an appetite for the vitamin itself, regardless of physical characteristics of the food.

The general plan of the experiments was to allow deficient animals their choice of 2 diets of distinctly different flavors, A (anise) and O (unflavored). The A flavored diet contained the vitamin, the O flavored diet contained none. After a few days the choices were changed so that the A flavored diet contained no vitamin but the O flavored diet did. If the animals continued to eat the A flavored diet when it now no longer contained vitamin, then the appetite was learned as defined by Harris et al. ('33), but if they now ate the O diet, the result was evidence of a true hunger.

In practice, this simple scheme was considerably modified to eliminate several possible variables: (1) While we have as yet found no sex differences in self selection behavior, these possible differences were eliminated by dividing the animals according to sex as evenly as possible. (2) Although previous experiments (Scott and Quint, '46a) had indicated that there was no appetite in rats for anise flavor, this possibility was eliminated by giving the vitamin-containing food to half the animals as a choice accompanied by flavor and to the other half unaccompanied by flavor at the start of the experimental period. (3) The same experiments had indicated that there was no effect of previous diet on appetite for anise. This pos-

sibility was eliminated, however, by feeding half the deficient animals an anise-flavored, vitamin-free diet and the other half an unflavored vitamin-free diet in the control period and by feeding half the normal animals a flavored vitamin-containing diet and the other half a similar diet but unflavored in the control period. (4) The flavor was changed with respect to the vitamin not once but 3 times. This gave additional data, particularly with respect to the extent and persistence of the learned eating process. (5) A group of normal animals was included in the experiment to serve as controls, and thus demonstrate clearly the effect of deficiency on the results. (6) The data obtained with all deficient animals were averaged as were the data with all normal animals. This resulted in the cancellation of the effects, if any, of sex, flavor, and previous diet as such, and allowed the uncomplicated estimation of the effect of deficiency in influencing association of some property of the vitamin with flavor of the food.

In each of 3 experiments, with thiamine, riboflavin, and pyridoxine, respectively, 10 male and 10 female weanling rats were therefore divided into 4 groups. Each group was fed during a 3-week control period 1 of the following diets in both of 2 cups: (1) standard diet,<sup>2</sup> (2) standard diet plus flavor, (3) standard diet plus vitamin, (4) standard diet plus vitamin plus flavor. Other vitamins were fed separately as pills.<sup>3</sup> During this 3-week period, the cups were alternated in a pre-determined random manner, and the amount of food eaten from each cup was recorded daily. At the end of the period,

<sup>2</sup>The standard diet consisted of 24% "Labco vitamin-free" casein, 10% "Primex," 62% sucrose, and 4% salt mixture (Jones and Foster, '42). Flavored diets contained 10 p.p.m. oil of anise. Vitamin-containing diets contained 5  $\mu\text{g}/\text{gm}$  vitamin in the case of thiamine and pyridoxine, and 10  $\mu\text{g}/\text{gm}$  in the case of riboflavin.

<sup>3</sup>One pill was given each rat daily. It contained approximately: 60  $\mu\text{g}$  thiamine hydrochloride, 120  $\mu\text{g}$  riboflavin, 90  $\mu\text{g}$  pyridoxine hydrochloride, 150  $\mu\text{g}$  calcium pantothenate, 10 mg choline chloride, 1 mg  $\alpha$ -tocopherol, and 55 I.U. vitamin A, and 11 I.U. vitamin D as 0.001 ml Natola, all in a dextrin-powdered sugar base. In the thiamine experiment, thiamine was omitted from the pills; in the riboflavin experiment, riboflavin was omitted; in the pyridoxine experiment this vitamin was omitted.

half of the animals were suffering from severe vitamin deficiency while the other half were considered to be normal.

The 3-week experimental period was broken into 4 parts, during which half of the deficient animals received choices shown in column (1) of table 1 and half received the choices shown in column (2). Similarly, half of the normal animals received the choices in column (1) and half those in column (2). The cups were alternated as before and the amount of food eaten from each cup recorded daily.

TABLE 1  
*Plan of experimental period.*

PART	LENGTH OF PERIOD DAYS	CHOICES	
		(1)	(2)
First	5	Unflavored, no vitamin; flavored, vitamin-containing	Flavored, no vitamin; unflavored, vitamin-containing
Second	5	Flavored, no vitamin; unflavored, vitamin-containing	Unflavored, no vitamin; flavored, vitamin-containing
Third	5	Same as first period	Same as first period
Fourth	6	Same as second period	Same as second period

## RESULTS

As was anticipated from previous experiments, at the start of the experimental period in all 3 experiments the deficient animals ate the vitamin-containing diet primarily, while the normal animals showed little preference. In the second part of the experimental period, the deficient animals ate chiefly of the vitamin-free diet, which was now flavored the same as the vitamin-containing diet had been in the first part of the experimental period. Normal animals for the most part again showed little distinctive preference. In the third part, choice was similar to what it had been in the first part, while in the last part, the results were variable.

The data are presented in tables 2 and 3, while the marked alternation of the amount of vitamin-containing diet eaten



TABLE 2  
*General analysis of results.<sup>1</sup>*

group	CONTROL PERIOD		EXPERIMENTAL PERIOD			
	Weight gain	Food eaten	Weight gain	Food eaten	Change in per cent eaten from cup I <sup>2</sup>	Per cent eaten associated with flavor <sup>3</sup>
	gm	gm	gm	gm		
Deficient Normal	14.1 ± 3.2	95.6 ± 4.2	Thiamine		17.2 ± 5.3	70.3 ± 7.0
	74.8 ± 2.2	166.4 ± 5.4			-2.8 ± 4.4	49.5 ± 7.5
Deficient Normal	10.2 ± 1.8	79.2 ± 5.3	Riboflavin		25.4 ± 4.2	63.9 ± 3.6
	67.0 ± 3.8	156.8 ± 9.0			12.5 ± 4.1	45.5 ± 5.6
Deficient Normal	18.4 ± 2.3	92.3 ± 5.4	Pyridoxine		22.3 ± 6.5	67.2 ± 6.5
	59.2 ± 2.9	134.6 ± 6.9			1.3 ± 6.9	55.7 ± 4.4

<sup>1</sup> All results in terms of the mean and standard of the mean.

<sup>2</sup> Control period minus experimental period. The vitamin-containing food was in all cases in cup II in the experimental period, and thus a positive change in per cent eaten from cup I indicates an overall preference for the vitamin-containing food.

<sup>3</sup> Per cent eaten of that food, flavored or unflavored, which first contained the vitamin in the experimental period. A result significantly greater than 50 indicates an association of flavor with beneficial effect of the vitamin.

TABLE 3  
*Detailed analysis of experimental period.*

Group	FIRST PART		SECOND PART		THIRD PART		FOURTH PART	
	Weight gain	Per cent eaten <sup>1</sup>	Weight gain	Per cent eaten	Weight gain	Per cent eaten	Weight gain	Per cent eaten
	gm/day		gm/day		gm/day		gm/day	
				Thiamine				
Deficient	3.18 ± .51	94.0 ± 3.2	2.46 ± .64	35.8 ± 11.5	3.51 ± .40	85.3 ± 8.2	4.20 ± .28	46.5 ± 11.9
Normal	3.48 ± .24	36.8 ± 7.6	3.62 ± .31	46.8 ± 9.1	3.08 ± .36	51.9 ± 8.7	2.95 ± .37	47.6 ± 6.6
				Riboflavin				
Deficient	4.36 ± .42	79.5 ± 9.0	3.22 ± .30	46.1 ± 8.1	3.32 ± .36	40.2 ± 5.0	2.62 ± .48	58.6 ± 6.8
Normal	2.74 ± .48	46.7 ± 12.3	3.80 ± .44	79.7 ± 6.9	2.86 ± .28	70.1 ± 8.5	2.48 ± .30	55.0 ± 7.5
				Pyridoxine				
Deficient	4.28 ± .32	78.1 ± 10.5	2.58 ± .28	35.9 ± 12.8	3.50 ± .22	90.3 ± 6.2	2.33 ± .25	63.4 ± 7.3
Normal	3.14 ± .26	57.2 ± 8.0	2.56 ± .24	56.2 ± 9.3	2.42 ± .20	56.3 ± 9.6	2.08 ± .23	39.7 ± 4.7

<sup>1</sup> Per cent of food eaten from the diet containing the vitamin.

by deficient animals with change in flavor is shown in figure 1. It should be noted from table 3 that the deficient animals failed to gain as much weight during the second part of the experimental period when their choice of diet with respect to the vitamin was poor as they did during the first and third parts when they ate primarily the vitamin-containing diets. From table 2 it is seen that the deficient animals had significant tendencies both to eat that diet, flavored or unflavored, which had contained the vitamin at the start of the experi-

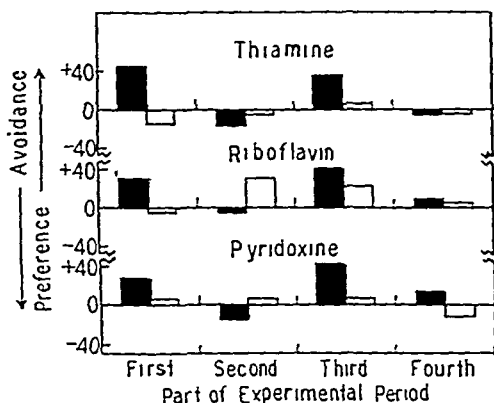


Fig. 1 Effect of alternation of flavor on appetite for vitamin-containing foods. Flavor was alternated with respect to the vitamin between each part of the experimental period. Preference (positive) or avoidance (negative) was calculated as per cent eaten of vitamin-containing food minus 50. Solid bars—average of 10 deficient animals; open bars—average of 10 normal animals.

mental period, and to eat more of the vitamin-containing diet than of the vitamin-free diet. Further analysis of the data indicated that there was no preference or avoidance of anise flavor as such and that there were no evident sex differences in eating behavior.

#### DISCUSSION

The alternation in amount eaten of vitamin-containing diet with change in flavor shown by deficient animals in figure 1, and the significant tendency of these animals to eat more of the diet of that flavor originally associated with the vitamin,

are proof of an association by the deficient animals of some deficiency-relieving quality of the vitamin with flavor or lack of it. This association must have involved a learning process, since flavor or lack of it was an artificial, not an inherent, characteristic of the vitamin. Thus, the appetites shown by rats for thiamine, riboflavin, and pyridoxine appeared to be learned appetites as proposed by Harris et al. ('33), and not true hungers. Harris concluded that the production of a feeling of well-being in the deficient animal was that quality of the vitamin-containing diet which was responsible for the animals' choice. This was, of course, an assumption, since one cannot easily determine what a rat feels. No other explanation seemed probable, however.

As is evident from figure 1, the tendency of the deficient animals to eat more of the vitamin-containing diet than of the vitamin-free diet during the experimental period as a whole was caused by incomplete change from the vitamin-containing diet to the vitamin-free diet when the flavors were switched after the first and third parts of the experimental period. The rats did not eat the vitamin-free diet to as great an extent nor for as long in the second and fourth 5-day periods as they had eaten the vitamin-containing diet in the 2 previous 5-day periods. This was not primarily due to an association of the effects of the vitamin with something other than flavor (e.g., flavor of the vitamin itself), because the eating behavior of only 3 out of the 30 deficient animals used in these experiments could possibly be interpreted in this light (i.e., only 3 animals did not exhibit at least a slight tendency to associate the vitamin with flavor or lack of it). Nor was it due to a relearning process, in which the vitamin became associated with the new flavor in the second and fourth periods, because when the original choices were offered in the third part of the experimental period, the preferences for the vitamin-containing diet were as strong as or stronger than in the first part.

The explanation was to be found primarily then in lack of stimulus to eat the vitamin-free diet. There must be 2 reasons

why deficient animals in the usual experiment continue to have an appetite for the vitamin-containing diets. One is that a stimulus to eat it (presumably the derived feeling of well-being) exists over a period of time; and in these experiments it must have existed over a period of at least 15 days. The other is that a habit is set up which makes customary the eating of a diet with a particular recognizable physical characteristic—in this case, flavor, or lack of it. A clear explanation of our results is then possible. During the first and third parts of the experimental period, both stimulus and habit contrived toward the eating of the vitamin-containing diet, but during the second and fourth parts habit exerted its influence in favoring choosing of the vitamin-free diet but stimulus was lacking. Consequently, the shift from vitamin-containing to vitamin-free diets when flavor was switched the first and third times was incomplete.

It has been found in 2 experiments on riboflavin deficiency, 1 of which is reported here, that animals previously receiving riboflavin have a slight but definite appetite for riboflavin. This appetite was significantly less obvious than that found in animals previously fed a diet deficient in riboflavin, and it was not apparent in our first published experiment on riboflavin (Scott and Quint, '46b). This appetite accounts for the unusual behavior of the normal group in the riboflavin experiment when compared with the normal groups in the thiamine and pyridoxine experiments. During the second and third parts of the experimental period, the normal animals preferred the riboflavin-containing diet to a certain extent, but there was no evidence of an association by this group of preference for riboflavin with flavor.

#### SUMMARY

The data obtained in these experiments are interpreted as meaning that the appetites for thiamine, riboflavin, and pyridoxine shown by animals previously fed diets deficient, respectively, in these vitamins are learned, probably as a result of beneficial experience. It is suggested that in the

usual experiment, deficient animals continue to eat a vitamin-containing diet for 2 reasons: (1) A habit is set up involving association of a feeling of well-being with some characteristic of the diet; and (2) a stimulus to eat the diet, presumably the derived feeling of well-being, persists over a considerable period of time. Animals previously fed a diet containing riboflavin showed a slight but definite preference for a riboflavin-containing diet.

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# THE CORNEAL VASCULARIZATION RESULTING FROM DEFICIENCIES OF AMINO ACIDS IN THE RAT<sup>1</sup>

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NINE FIGURES

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The association of corneal vascularization in man with nutritional deficiencies has led to a study of this phenomenon in experimental animals. Among the various deficiencies which will result in corneal vascularization in the rat are protein deprivation (Hall et al., '46) and deficiencies of lysine, tryptophane (Totter and Day, '42), methionine (Berg and co-workers, '47), leucine and histidine (Maun, Cahill and Davis, '45b, '46). In phenylalanine deficiency Maun, Cahill and Davis ('45a) found that "neither the eyes of the deficient, nor those of the control animals, showed alterations." Sydenstricker and associates ('46) have suggested that corneal vascularization may be a reaction resulting from a deficiency of any of the indispensable amino acids, or of protein. It thus seemed desirable to determine whether deficiencies of other amino acids resulted in changes in the cornea.

## METHODS

Twenty-five or 26-day-old rats of a Wistar strain were placed in individual cages and given a control diet or one de-

<sup>1</sup> A preliminary report of this work was published in the *Proc. Soc. Exp. Biol. Med.*, 64: 59, 1947.

ficient in phenylalanine, isoleucine, leucine, arginine, histidine, threonine or valine. The details of the methods and techniques used and the care of the animals were as previously described (Bowles et al., '46). As a source of protein in the control diet, the amino acid mixture of Maun, Cahill and Davis ('45a) was used, which supplied approximately 3 times the minimum required amount of each of the indispensable amino acids in utilizable form. The amino acid mixture used in the deficient diets was identical with this except that in each deficient diet the appropriate amino acid in the mixture was replaced with sucrose. The remainder of each diet contained in 100 gm: salt mixture<sup>1</sup> 4 gm, cod liver oil 2 gm, cottonseed oil 3 gm, choline chloride 0.2 gm, riboflavin 1.6 mg, thiamine hydrochloride 0.4 mg, calcium pantothenate 2 mg, pyridoxine hydrochloride 0.4 mg and sufficient sucrose to make 100 gm.

Para-aminobenzoic acid and inositol were not included in the diet since they appear to favor synthesis of amino acids by the intestinal flora (Hall and Sydenstricker, '47). The corneas of rats fed control diets to which were added only the vitamins as used in the present study showed no significant abnormality either on examination with the biomicroscope or in histological sectioning (Berg et al., '47).

One litter of 8 rats, 8 litters of 6 rats each, 3 litters of 5 rats, 2 litters of 4 rats and 1 litter of 3 rats were distributed among the diets so that the number of rats on each diet was as shown in table 1. No 2 rats from any litter were on the same diet.

In an attempt to produce more extensive corneal changes in rats deficient in threonine and valine, 3 rats were placed on each of 3 diets which were similar to the control diet except that the first contained only 0.30% of DL-valine, the second 0.20% of DL-threonine and the third 0.35% of DL-threonine. One rat from each group where corneal vascularization occurred was changed to the control diet to induce regression of vascularization. Two or 3 rats from these groups having

<sup>1</sup> The salt mixture was the same as that used by McKibben, Madden, Black and Elvehjem ('39).



marked corneal vascularization typical of the group as shown by biomicroscopic examination were selected for injection and subsequent photography of the cornea. Biomicroscopic examinations of the rats' eyes were made 4 times weekly with most of the rats and 3 times weekly for the remainder.

As before, we limit our use of the term "corneal vascularization" to the situation where there is an actual development of capillaries in the cornea beyond the limits of normal variation.

TABLE 1

*Corneal vascularization and growth of experimental rats.*

DIET	NO. OF RATS IN GROUP	INCIDENCE OF CORNEAL VASCULARI- ZATION	DAYS REQUIRED FOR APPEARANCE OF CORNEAL VASCULARIZATION		AVERAGE CHANGE IN WT. PER DAY DURING 30 DAYS
			Range	Average	
Control	8	0			gm + 1.2
Phenylalanine-deficient	11	11	3-13	7	- 0.6
Leucine-deficient	11	10	3-19	11	- 0.6
Isoleucine-deficient	11	11	4-11	8	- 0.7
Arginine-deficient	8	7	4-20	11	+ 0.7
Histidine-deficient	13	11	4-34	15	- 0.3
Threonine-deficient	11	9	3-36	19	- 0.5
Valine-deficient	9	9	3-20	11	- 0.8

Such normal variation is remarkably slight. An occasional rat, certainly less than 1 in a hundred, will have scleral flaps carrying aberrant portions of the limbic plexus which extend slightly beyond the usual periphery of the cornea. Even less frequently, accidental trauma to a cornea may cause confusing corneal changes.

## RESULTS AND DISCUSSION

Quite regularly, the first sign of ocular reaction to deficiency of amino acids or of protein is congestion of the scleral conjunctiva and engorgement of the limbic plexus. Soon thereafter, often concurrently, there is slight thickening and diffuse clouding of the cornea, probably due to edema. Subsequently,

short capillary "sprouts" can be seen which derive from the marginal vessels of the limbic plexus. It is thought that these actually are the afferent limbs of small capillary loops. Such "sprouts" increase in length and caliber and soon their efferent limbs are visible. As such capillary loops invade the subepithelial region of the cornea, anastomotic vessels are apt to develop so that the entire cornea may become shrouded in a dense network of centripetally growing vessels in which no definite pattern can be made out.

Corneal opacity due to edema and leukocytic infiltration tends to parallel the severity of vascular invasion. At times, diffuse opacity may prevent accurate evaluation of the degree of vascularity present. In some animals scattered nebular opacities precede the general clouding. The grade of vascularization, its pattern and the severity of the associated opacity vary much in different deficiencies. With deficiency of total protein, or of phenylalanine, leucine or isoleucine, corneal vascularization and opacity may be extreme so that the tissue seems to be converted into an area of granulation tissue covered by finely wrinkled, dry, sometimes ulcerated, epithelium. This we have also observed to be the result of tryptophane deficiency.<sup>3</sup> In deficiency of histidine, valine, or threonine the invading vessels remain small and their radial arrangement seldom is disturbed. Corneal opacity may be very slight so that the newly formed vessels are visualized with little difficulty and no abnormality of the corneal epithelium can be seen. This is similar to what we have observed to occur in methionine deficiency (Berg et al., '47). Rats deficient in phenylalanine and lysine<sup>3</sup> have shown some variation in the grade of keratitis they developed but extremes have not been observed.

The rats fed diets devoid of valine and threonine usually died as early corneal changes appeared. In the 3 rats fed 0.30% of DL-valine the rats lived long enough to develop more extensive corneal changes and vascularization. This diet supplied 0.15% of L-valine as compared to the 0.7% of

<sup>3</sup> Unpublished data.

this form of valine required for growth (Rose, '37). The rats fed 0.35% and those fed 0.20% of DL-threonine developed congestion of the sclera and a slight degree of corneal opacity but no corneal vascularization. These 2 groups of rats received, respectively, 0.17% and 0.10% of L-threonine, the form utilizable for growth, as compared to the rats' requirement of 0.5% for growth (Rose, '37).

The rats in the group fed the arginine-deficient diet developed only a very minor degree of corneal vascularization, which later spontaneously regressed. One of the control rats developed a few capillary loops into the edge of the cornea beyond the limits of normal variation. The corneas of the remaining control rats appeared normal except for an occasional slight translucency of the cornea which would later disappear. Maun, Cahill and Davis ('45a) noted no histological changes in the eyes of their control rats fed a diet similar to this.

When the deficient rats were returned to the control diet to supply the lacking essential amino acid, prompt regression of the vascularization occurred until corneal capillaries could no longer be seen with the biomicroscope. The time required for complete regression varied somewhat, depending on the degree of corneal vascularization.

The incidence and time of appearance of vascularization in the groups of rats and growth data are given in table 1. It may be seen that vascularization appeared most rapidly in phenylalanine deficiency and most slowly in threonine deficiency.

All the rats on the deficient diets lost weight, except for the arginine-deficient rats which grew at a rate of 0.7 gm per day. The growth of 1.2 gm per day for the control rats may be compared with the rate of about 3.0 gm per day usual for our strain of rats at this age when receiving an adequate diet. Since the protein requirement of the rats was supplied with amino acids, a subnormal growth would be expected, as this diet would lack the growth factor for rats described by Womack and Rose ('46) as present in intact and partially

hydrolyzed proteins. Taking into consideration the difference in rate of normal growth of the strains of rats used by Womack and Rose ('46) and those used in the present study, the diets used by Womack and Rose, which contained the non-essential amino acids, resulted in considerably better growth than did our control diet which lacked these amino acids. With the chick, Luckey and associates ('47) obtained superior growth with diets containing some of the non-essential amino acids as compared with diets containing only the amino acids now recognized as essential for the chick.

Plates 1 and 2 show reproductions of photographs of injected corneas illustrating the type of vascularization obtained in each of the deficiencies studied. It will be noted that while there are differences in pattern of vascularization in the deficiencies studied here, and in those previously studied, these differences in no case are very striking and the patterns of vascularization in several of the deficiencies are indistinguishable. The observations which we made in earlier studies as to development of pattern of vascularization also apply here.

The appearance of enlarged distal ends of developing capillaries in threonine, leucine and phenylalanine deficiencies is similar to what we had observed before in protein deprivation. The pictures of injected corneas here may be compared with the pictures of the injected corneas of normal rats shown in an earlier paper (Bowles et al., '46).

Our observations that phenylalanine deficiency will cause corneal vascularization are not in agreement with the statement of Maun, Cahill and Davis ('45a) that they observed no alteration of the eyes of their phenylalanine-deficient animals. It is possible that this discrepancy may be explained as being a strain difference since Maun, Cahill and Davis used Sprague-Dawley animals while rats of a Wistar strain were used in the present study.

#### SUMMARY

Deficiencies of phenylalanine, isoleucine, threonine, valine and arginine were found to result in corneal vascularization.

The corneal vascularization resulting from leucine and histidine deficiencies was also studied. It now seems clear that lack of protein or of any 1 of the 10 essential amino acids may result in corneal vascularization in the rat. Other changes observed in varying degrees were diffuse and nebular corneal opacity, and edema of the cornea.

#### ACKNOWLEDGMENT

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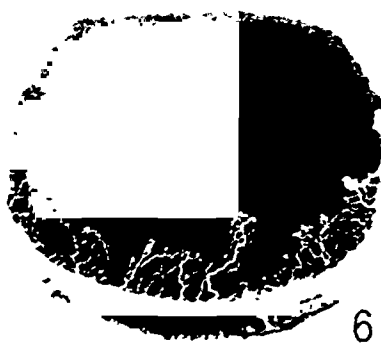
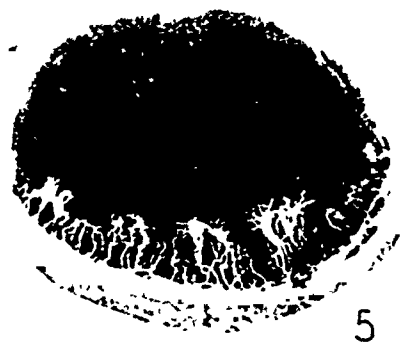
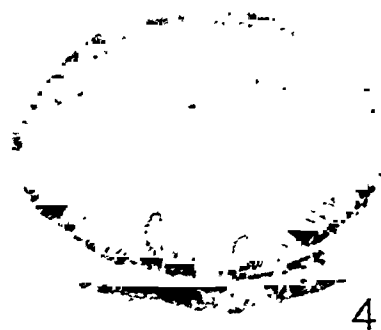
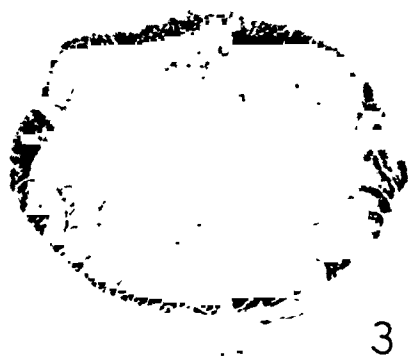
## PLATE 1

## EXPLANATION OF FIGURES

(All 18 X reduced approximately one-third)

Oblique views of India ink-injected corneas of rats fed amino acid-deficient diets.

- 1 Histidine-deficient diet. Corneal vascularization observed after 16 days, rat killed after 33 days on the diet.
- 2 Histidine-deficient diet. Corneal vascularization after 16 days, rat killed after 33 days on the diet.
- 3 Low-valine diet (0.30% of DL-valine). Corneal vascularization after 13 days, rat killed after 54 days on the diet.
- 4 Valine-free diet. Corneal vascularization after 19 days, rat killed after 23 days on the diet.
- 5 Threonine-deficient diet. Corneal vascularization after 25 days, rat killed after 46 days on the diet.
- 6 Isoleucine-deficient diet. Corneal vascularization after 11 days, rat killed after 49 days on the diet.





(All  $18\times$  reduced approximately one-third)

Oblique views of India ink-injected corneas of rats fed amino acid-deficient diets.

7 Leucine-deficient diet. Corneal vascularization observed after 8 days, rat killed after 29 days on the diet.

8 Phenylalanine-deficient diet. Corneal vascularization after 5 days, rat killed after 51 days on the diet.

9 Arginine-deficient diet. Corneal vascularization after 4 days, rat killed after 81 days on the diet.



# THE PROTEIN REQUIREMENTS OF THE ADULT RAT IN TERMS OF THE PROTEIN CONTAINED IN EGG, MILK AND SOY FLOUR<sup>1</sup>

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ONE FIGURE

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Recently several studies have been reported of the protein value of foods for mature animals using a method proposed by Melnick and Cowgill ('37) and demonstrated with dogs. The method involves the use of animals in negative nitrogen balance at various levels of nitrogen intake, and receiving adequate caloric intakes. Under such conditions the relationship between nitrogen intake (or absorbed nitrogen) and nitrogen balance is a linear one. The quality of the protein as well as the quantity needed to maintain nitrogen equilibrium can be estimated from the mathematical description of the regression line describing the relationship. This method was used by Harris and Mitchell ('41) to study the utilization of urea nitrogen in sheep and was also employed by Allison and Anderson ('45) to study the quality of various proteins fed to dogs. The method was adapted to human subjects in a study of protein requirements in terms of different foods by Bricker, Mitchell and Kinsman ('45) and later, in a modified form, by Hegsted and coworkers ('46) who applied it to mixed diets. The present report deals with an application of this method to adult male rats.

<sup>1</sup> The data reported in this paper were taken in part from a thesis presented by Mildred Bricker to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

## EXPERIMENTAL PROCEDURE

Ten adult male albino rats were fed low-protein diets at 2 levels of intake in amounts sufficient to maintain body weight and energy equilibrium. The food protein sources, dried defatted whole egg, dried skim milk and soy flour (low-fat, expeller process) were each fed at one level estimated to give a slightly negative nitrogen balance and then at a lower level.

TABLE 1  
*Composition of experimental diets.*

	DIET A	DIET B	DIET C	DIET D	DIET F	DIET G
Starch	31.28	29.91	27.21	22.86	23.57	19.81
Cerelose	40.00	40.00	40.00	40.00	40.00	40.00
Vitamin supplement <sup>1</sup>	5.00	5.00	5.00	5.00	5.00	5.00
Mineral salts	4.00	4.00	4.00	4.00	4.00	4.00
Sodium chloride	1.00	1.00	1.00	1.00	1.00	1.00
Wood flock	4.00	4.00	4.00	4.00	4.00	4.00
Lard	9.65	9.18	9.94	9.90	9.26	8.96
Wheat germ oil	0.50	0.50	0.50	0.50	0.50	0.50
Cod liver oil	1.50	1.50	1.50	1.50	1.50	1.50
Barium sulfate	1.00	1.00	1.00	1.00	1.00	1.00
Dried defatted whole egg	3.07	4.61				..
Dried skim milk			5.85	10.24		..
Soy flour <sup>2</sup>					10.17	14.23
Total	101.00	101.00	100.00	100.00	100.00	100.00
Percent protein ( $N \times 6.25$ )	2.38	3.38	2.38	4.02	5.15	7.14

<sup>1</sup> The vitamin supplement added in a 5% starch vehicle provides in 100 gm of the diet: 500  $\mu$ g thiamine, 650  $\mu$ g riboflavin, 300  $\mu$ g pyridoxine, 3 mg pantothenic acid, 400 mg choline, 60 mg nicotinamide and 500  $\mu$ g ascorbic acid.

<sup>2</sup> Low-fat expeller process soy flour obtained directly from the A. E. Staley Manufacturing Company, Decatur, Illinois.

For the egg protein, diets containing higher levels were employed, designed for each rat, on the basis of the data secured on the 2 lower levels, to support approximate nitrogen equilibrium. The composition of the diets, with the exception of the latter, may be found in table 1. An attempt was made to have all the diets similar and adequate in nutritive value in all respects except protein. The total amount of fat was kept at

12%, including the fat in the test foods. Barium sulfate was used as a filler so that on days when feces markers were given the barium sulfate could be replaced with ferric oxide.

The quantity of diet given each rat was sufficient for energy equilibrium as measured in a pre-feeding period of 11 days by maintenance of constant body weight. On each diet a 5-day preliminary adjustment period was followed by a 7-day collection period. Urine samples were preserved with dilute hydrochloric acid and pooled for the entire week. The week's feces samples were composited, dried at 110°C. and ground before analysis. Nitrogen analyses were made on the food and excreta by the macro-Kjeldahl method, using mercury as a catalyst.

#### EXPERIMENTAL RESULTS

The essential data from the metabolism studies with whole egg protein fed at the various dietary levels are summarized in table 2. The food intakes of the rats were essentially constant from period to period. With increasing levels of nitrogen intake, the fecal nitrogen per gm of food showed no significant change, averaging 1.21 mg. This circumstance shows that the nitrogen of the whole-egg preparation used in these experiments was completely digestible and that the fecal nitrogen is all of body (metabolic) origin, confirming the observations of Mitchell and Carman ('26b).

The daily urinary nitrogen per unit of metabolic mass ( $W^3$  gm) actually decreased with successive increases in nitrogen intake, averaging 0.826, 0.737 and 0.692 mg, respectively. The nitrogen balances become less negative and, eventually, positive. Comparing the average changes in nitrogen intake with the average change in nitrogen balance, it appears that an increase of 1 mg of nitrogen consumed is associated with a decrease in the loss of nitrogen to the body of 1.28 mg in passing from the lowest to the intermediate level of dietary protein, and of 1.38 mg of nitrogen in passing from the intermediate to the highest level of dietary protein. This anomalous relationship probably signifies a depression in the minimum

endogenous catabolism of nitrogen with continued experimentation involving prolonged periods of nitrogen depletion, analogous to the depression in the basal metabolism after long periods of inanition.

That this is not a specific effect of egg protein is indicated by the results of an earlier experiment on young rats. Two groups of 4 rats each were fed alternately, in successive (and

TABLE 2

*The nitrogen metabolism of the rats on 3 levels of whole egg proteins.*

RAT NO.	FECAL NITROGEN PER GM OF FOOD CONSUMED			URINARY NITROGEN PER W <sup>1</sup> GM			DAILY NITROGEN BALANCE		
	2.38% protein <sup>1</sup>	3.28% protein	3.84% protein <sup>2</sup>	2.38% protein	3.28% protein	3.84% protein <sup>2</sup>	2.38% protein	3.28% protein	3.84% protein <sup>2</sup>
	mg	mg	mg	mg	mg	mg	mg	mg	mg
1	1.20	1.09	1.09	0.87	0.63	0.60	-29	+ 6.8	+17
2	1.01	1.11	1.17	0.83	0.71	0.73	-22	+ 0.5	+ 9
3	1.19	1.26	1.20	0.75	0.64	0.63	-21	+ 2.8	+14
4	1.15	1.18	1.22	0.71	0.66	0.66	-17	+ 1.8	+11
5	1.03	1.10	1.16	0.71	0.76	0.69	-15	- 2.8	+14
7	1.22	1.28	1.09	0.72	0.67	0.58	-21	- 0.3	+20
8	1.36	1.33	1.11	0.74	0.66	0.61	-28	- 1.7	+20
11	1.17	1.26	1.34	1.00	0.97	0.83	-34	-17.1	+20
12	1.37	1.40	1.29	0.94	0.83	0.90	-37	-10.9	-0.4
14	1.33	1.31		1.00	0.85		-43	-10.7	..
Averages	1.20	1.23	1.19	0.826	0.737	0.692	-26.7	- 3.1	+13.9

<sup>1</sup> In these headings, the term protein refers to conventional protein =  $N \times 6.25$ . The metabolism tests were carried out from July 9 to 16, July 23 to 29 and August 29 to September 4, respectively.

<sup>2</sup> The diets used in this period varied in protein level from rat to rat from 3.59 to 4.62% (averaging 3.84), in an attempt to approximate the level required for nitrogen equilibrium.

continuous) 7-day metabolism periods, a 4% egg-protein diet and a nitrogen-free diet. The order of alternation differed in the 2 groups. The data are summarized in table 3. The rate of decline in urinary nitrogen per W<sup>1</sup> gm is again evident, but it is not appreciably affected by the order of feeding of the experimental diets. Specifically, the individual differences in the 2 groups of rats between the observed value for the

second period and the mean for periods 1 and 3 were not statistically distinct ( $P = \text{ca. } 0.27$ ).

This experiment shows also that the nitrogen of whole egg is completely utilized in the metabolism of the growing rat within the limits of accuracy of the methods used, confirming again the results of Mitchell and Carman ('26b). That this is also true for the adult male rat is demonstrated by the

TABLE 3

*The utilization of the nitrogen of whole egg by the growing rat. Successive 7-day collection periods.*

DIET	NO. OF RATS	AVERAGE BODY WEIGHT	DAILY FOOD AVERAGE	AVERAGE FECAL N PER GM OF FOOD	AVERAGE URINARY N PER W <sup>3</sup> GM	AVERAGE URINARY N PER W <sup>3</sup> GM FOR PERIODS 1 AND 3
		gm	gm	mg	mg	mg
Nitrogen — Low	4	57.1	3.34	1.74	1.138	
4% egg protein	4	55.6	3.39	1.98	.853	.910
Nitrogen — Low	4	52.7	2.62	1.41	.683	
4% egg protein	4	60.8	3.36	2.13	1.057	
Nitrogen — Low	4	56.1	3.25	1.44	.780	.888
4% egg protein	4	55.6	2.68	1.77	.719	

data collected in tables 4 and 5, taken from unpublished experiments in this laboratory (Treichler, '39). Both groups of rats subsisted for 14 days on the 4% egg-protein diet, collections of excreta being made for the last 7 days only. In this period the daily urines were composited for analysis. The rats were then put upon a nitrogen-free diet in which the dried defatted whole egg was replaced by sucrose, and the daily urine collections were analyzed separately with the results shown in the tables. All diets contained 22% of fat and approximately 4.6 cal. of gross energy per gm.

The rats yielding the data in table 4 averaged about 240 gm in weight. Their urinary nitrogen output per day was not significantly different on the 2 diets. Expressed per W<sup>3</sup> gm per day, the averages were 0.786 mg of nitrogen on the low egg-protein diet and 0.727 mg on the nitrogen-free diet. A

TABLE 4

*The utilization of the nitrogen of whole egg at low levels of intake by young mature rats at 28°C. on maintenance rations.<sup>1</sup>*

DAYS ON TEST	AVERAGE DAILY N INTAKE	DAILY EXCRETION OF URINARY NITROGEN				
		Rat 55	Rat 58	Rat 61	Rat 64	Rat 67
	mg	mg	mg	mg	mg	mg
4% egg-protein diet						
7	52	52	41	46	55	47
Low-nitrogen diet						
1	5	53	42	46	46	36
2	5	55	46	49	53	36
3	5	49	44	42	41	44
4	5			38	40	40
5	5			48	37	39
6	5			43	44	41
7	5			47	39	46
8	5			36	38	42
9	5			52	35	46

<sup>1</sup> Taken from the thesis of Ray Treichler ('39).

TABLE 5

*The utilization of the nitrogen of whole egg by older mature rats on maintenance rations at comfortable temperatures.<sup>1</sup>*

DAYS ON TEST	AVERAGE DAILY N INTAKE	DAILY EXCRETION OF URINARY NITROGEN						Average for 6 rats
		Rat 29	Rat 30	Rat 31	Rat 32	Rat 33	Rat 34	
	mg	mg	mg	mg	mg	mg	mg	mg
4% egg-protein diet								
7	64	77	70	70	86	72	58	72
Low-nitrogen diet								
1	5	63	49	47	56	65	50	55
2	5	70	46	61	54	61	61	59
3	5	75	50	50	63	61	53	59
4	5	85	51	90	57	62	62	68
5	5	68	54	53	60	74	49	60
6	5	75	57	65	59	55	49	60
7	5	65	42	55	54	60	51	54

<sup>1</sup> Taken from the thesis of Ray Treichler ('39).

statistical analysis of the differences for individual rats afforded no basis for suspecting a diet effect. The rats were in negative nitrogen balance in both periods.

The second group of rats (table 5) averaged 390 gm in weight, and here the urinary nitrogen on the egg-protein diet averaged higher for all 6 rats than that on the low-nitrogen diet, 0.821 mg per W<sup>1</sup> gm against 0.680 mg. The probability that a fortuitous combination of the uncontrolled factors in the experiment could have produced an average difference as large or larger than this, and in the same direction, is only 0.016 (Student, '08) and may be neglected. However, in this experiment all rats were in positive nitrogen balance on the egg-protein diet, averaging 19.7 mg per day, so that the urine may have contained nitrogen consumed in excess of body needs.

The experimental data in these 2 tables bring further support to the conclusion that whole-egg protein is utilized in the metabolism of the mature rat with little or no wastage (table 4), or at least they are not in contradiction necessarily with this conclusion (table 5). It is to be noted in particular that egg protein in no case depressed the urinary nitrogen level secured on a low-nitrogen diet, in contrast to the observations reported by Brush, Willman and Swanson ('47).

Since the data on fecal nitrogen presented in table 2 indicate clearly that the nitrogen of the preparation of dried defatted whole egg used in these tests is completely digestible, the metabolic nitrogen in the feces may be computed in tests with other proteins by using the factor 1.21 mg per gm of food. This procedure has been used in computing the true digestibility of the nitrogen of milk and of soy flour with results summarized in table 6. The average true digestibility of milk nitrogen for these mature rats was 95.3 and that for the soy flour nitrogen was 90.4.

On the basis of evidence already presented, a biological value of 100 was assumed for the egg proteins. Therefore, the average urinary nitrogen excretion on the first 2 egg periods was assumed to be the endogenous nitrogen. These

values may be expressed in relation to the basal metabolism, in order to compare them with those published by Smuts ('35). The surface area of each rat was, therefore, estimated by the Lee ('29) formula. A basal metabolic rate of 686 (S.D. 97) cal. per m<sup>2</sup> per 24 hours was then assumed. This figure is an average of 70 basal metabolism determinations found in the literature. In arriving at this figure, the surface areas of the rats were all computed, or recomputed, by the formula of Lee. This meant recalculation of the original values in

TABLE 6

*The true digestibility of the proteins (nitrogen) in milk and in soy flour for mature rats.*

RAT NUMBER	MILK PROTEINS		SOY FLOUR PROTEINS	
	True digestibility	Biological value <sup>1</sup>	True digestibility	Biological value <sup>1</sup>
	%	%	%	%
1	93	96	93	51
2	95	87	88	48
3	94	90	92	51
4	97	89	91	45
5	94	88	88	50
7	94	83	89	53
8	98	82		
11	94	96	90	62
12	96	75	92	56
14	98	100		
Averages	95.3	88.6	90.4	52.0

<sup>1</sup> Computed by assuming the endogenous nitrogen outputs in column 5 of table 7, and proceeding in accordance with the method of Mitchell ('24).

some cases. Only data for adult male rats were taken, including 24 basal metabolic rate determinations by Mitchell and Carman ('26a), 15 determinations by Smuts ('35), 26 by Benedict, Horst and Mendel ('32) and 5 by Treichler and Mitchell ('41).

Estimates of surface area and basal caloric expenditures of the rats in this experiment are tabulated in table 7. The endogenous nitrogen in the urine is expressed in mg per basal calorie, and averages for all 10 rats  $2.162 \pm 0.120$ . This value



agrees well with that of  $1.988 \pm 0.034$  mg reported by Smuts ('35) for adult rats.

This experiment was designed on the assumption that a linear relationship exists in the mature rat between the absorbed nitrogen and the nitrogen balance within the area of negative nitrogen balances. In figure 1 this relationship is shown graphically. Values for individual animals are expressed in relation to basal calories in accordance with the

TABLE 7

*The weight, surface area, basal calories, and endogenous nitrogen of the adult male rats used in these experiments.*

RAT NO.	BODY WEIGHT	SURFACE AREA <sup>1</sup>	BASAL METABOLISM <sup>2</sup>	ENDOGENOUS NITROGEN	
	gm	cm <sup>2</sup>	cal./day	mg/day	mg per basal cal.
1	312	393	26.96	55.05	2.042
2	313	394	27.03	56.44	2.085
3	324	402	27.58	53.00	1.922
4	326	404	27.71	52.50	1.895
5	314	395	27.10	54.75	2.020
7	364	432	29.64	57.66	1.945
8	408	462	31.69	63.66	2.009
11	294	380	26.07	69.66	2.672
12	334	410	28.13	69.10	2.456
14	336	411	28.19	72.51	2.572
Average				2.162 $\pm$ 0.120	

<sup>1</sup> Calculated by the Lee formula,  $S_{cm^2} = 12.54 W_{gm}^{.60}$

<sup>2</sup> Assuming a basal metabolic rate of 686 cal. per square meter per 24 hours.

Terroine ('27)-Smuts ('35) principle. Regression lines were then fitted to the data by the method of least squares. The equation,  $Y = a + bX$  was used, in which  $Y$  represents the nitrogen balance,  $X$  the intake of absorbed nitrogen,  $a$  the intercept on the  $X$  axis, i.e., the amount of nitrogen excreted in the urine and feces when no nitrogen is consumed, and  $b$  the rate of increase in  $Y$  as  $X$  increases, or the biological value of the protein, all values being expressed per basal calorie.

The resulting equations are:

$$Y = 0.990 X - 2.757 \text{ for egg proteins,}$$

$$Y = 0.857 X - 2.727 \text{ for milk proteins, and}$$

$$Y = 0.494 X - 2.711 \text{ for soy flour proteins.}$$

The biological values (the  $b$  constants) indicated by these equations, expressed as percentages, are 99 for egg proteins, 86 for milk proteins and 49 for soy proteins, slightly less than the values that may be computed for the individual rats on the assumption of a biological value of 100 for egg proteins. These individual values have been summarized in table 6.

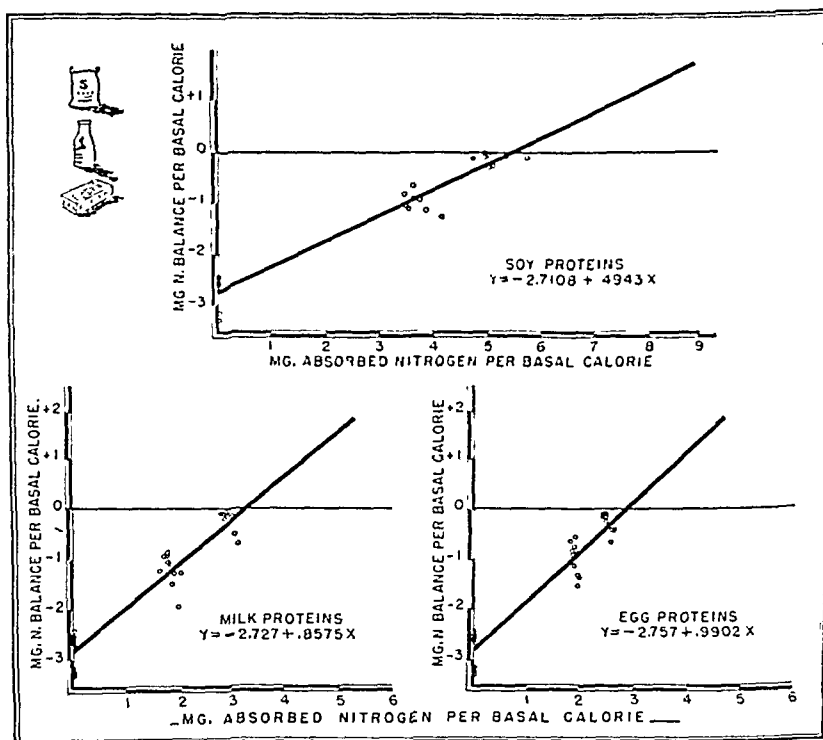


Fig. 1 The relationship between the truly absorbed nitrogen ( $X$ ) and the nitrogen balance ( $Y$ ) on the different experimental diets. In the equations given, the  $a$  value represents the nitrogen excretion at 0 intake and the  $b$  value, the slope of the line, or the biological value of the protein. The points at 0 intake are the average fecal plus urinary nitrogen excretion on the 2 low-egg periods.

The amount of absorbed nitrogen required for equilibrium may be calculated by solving for  $X$  when  $Y=0$ . Thus, in terms of truly absorbed nitrogen, 2.784 mg of egg nitrogen per basal calorie, 3.179 mg of milk nitrogen and 5.484 mg of soy-flour nitrogen are needed to maintain nitrogen equilibrium in the rats studied.

The amounts of total food nitrogen needed to maintain nitrogen equilibrium can be calculated by dividing the requirement of truly absorbed nitrogen by the true digestibility coefficient expressed as a decimal and are as follows: 2.784 mg of egg nitrogen per basal cal., 3.337 for milk and 6.066 for soy flour. When these nitrogen values are converted to the necessary per cents of protein in the diets used in this experiment they become 3.60 for egg, 4.33 for milk and 7.61 for soy proteins on the dry basis.

#### DISCUSSION

In similar studies by the authors (Bricker, Mitchell and Kinsman, '45) on human subjects a value of 2.76 mg of total milk nitrogen per basal cal. was needed for the maintenance of nitrogen equilibrium. This is to be compared with the value, on the same basis, of 3.34 mg for the rats in this study. If both requirements are computed on a weight basis, that for the human is 65 mg of nitrogen per kg and for the rat, 229. The agreement between the former values, as well as the disagreement between the latter is in harmony with the conclusions of Terroine ('27) and of Smuts ('35) that protein requirements for maintenance are related to surface area and basal metabolism rather than to body weight as is all too commonly assumed.

The corresponding values for soy-flour nitrogen are 2.88 mg per basal cal. for the human adult and 6.07 for the adult rat. The difference in this case is large. It may be due, but only in small part, to a difference in heat treatment of the soy flours fed to the 2 species. It is most likely largely traceable to a difference between the rat and man in the relative requirements for sulfur-containing amino acids that limit the bio-

logical value of soy flour proteins (Johnson et al., '47; Cox et al., '47), for the rat at least.

Barnes, Bates and Maack ('46), employing essentially the same technic as that used in this study, reported that mature male rats require for the maintenance of nitrogen equilibrium 11.2 mg of apparently absorbed egg nitrogen per day per 100 cm<sup>2</sup> of body surface and, for 2 different soy flours, 13.6 and 15.1 mg of apparently absorbed nitrogen. Using the Lee formula ('29) for the estimation of surface area rather than that of Carman and Mitchell ('26), and assuming a body weight of 325 gm, raises these requirements to 14.9, 18.1 and 20.1 mg N per 100 cm<sup>2</sup>. The values obtained in this experiment are 19.0 mg of truly digestible egg nitrogen and 41.2 mg of truly digestible soy flour nitrogen per 100 cm<sup>2</sup> of body surface, or in terms of apparently digestible nitrogen, assuming 1.2 mg metabolic fecal N per gm of food (table 2), 15.0 mg egg N and 37.2 mg of soy flour N. The former value agrees well with the corresponding value of Barnes and associates, but the latter figure is considerably higher than the highest of the 2 values for soy flour reported by these investigators.

The biological value of 86 for milk protein agrees well with that of 86 determined by Boas-Fixsen and Jackson ('32) on 4 mature rats fed roller-process dried milk at a 7% protein level, and less well with that of 78 on mature rats fed at a 5% protein level by Sumner ('38). Sumner ('38) in the same study reports a value of 94 for egg proteins fed to mature rats at a 5% level of protein. Henry, Kon and Rowland ('46) obtained a biological value of 73 for milk protein for mature rats at an 8% level, a level too high to afford any assurance that dietary nitrogen is not wasted in metabolism merely because it is consumed in amounts exceeding requirements.

#### SUMMARY AND CONCLUSIONS

Ten adult male rats were fed 2 dietary levels each of milk and soy flour proteins and 3 dietary levels of egg protein; all of the former and 2 of the latter dietary levels were asso-

ciated with negative nitrogen balances. The linear relationship between truly absorbed nitrogen and nitrogen balance was described by regression equations fitted to the data by the method of least squares.

The amount of truly absorbed nitrogen (corrected for the metabolic nitrogen in the feces) required for nitrogen equilibrium was computed from these regression equations to be 2.78 mg of egg nitrogen, 3.18 mg of milk nitrogen and 5.48 mg of soy flour nitrogen per cal. of basal heat. The biological values indicated by these regression equations are 99 for whole egg nitrogen, 86 for milk nitrogen and 49 for soy flour nitrogen.

The nitrogen of the defatted dehydrated whole egg preparation used in these studies was completely digestible by the adult rat and, at the low levels of feeding used (2.38 to 4.62% conventional protein) the absorbed nitrogen was practically completely utilized in adult rodent metabolism. Neither in this experiment, nor in previous ones on adult rats carried out in this laboratory, has egg protein been observed to depress appreciably the minimum endogenous metabolism of nitrogen.

The total requirements of milk nitrogen per basal cal. for nitrogen equilibrium in the adult rat and the adult human are not greatly different, being 3.34 mg and 2.76 mg, respectively. The requirements of soy flour nitrogen on the same basis are much greater for the rat than for the human, 6.07 mg and 2.88 mg, respectively, testifying to the greater relative requirement of the rat than of the human for the amino acid severely limiting the nutritive value of soybean protein, namely, methionine (or cystine).

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# A COMPARISON OF THE UTILIZATION AND ACCEPTABILITY OF FRESH AND DEHYDRATED FOOD<sup>1</sup>

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The increasing use of dehydrated foods, particularly during World War II, would seem to make desirable a complete study of their nutritive value. The prolonged periods over which such foods may be stored without spoilage as compared with fresh foods make them especially adaptable for army use. This property also is of importance in the use of dehydrated foods for relief overseas. The lowered bulk combined with the great decrease in weight of the water-free food makes the shipment of much larger amounts of food possible with a limited amount of shipping space.

<sup>1</sup>This work was done under a contract between the University of Southern California and the Research and Development Branch, Military Planning Division of the Office of the Quartermaster General, U. S. A.

The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions and conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

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<sup>3</sup>We wish to thank Mr. W. R. Harriman, Director, and Dr. Douglas W. Ritchie, Medical Director, for making the arrangements essential to this study; and to acknowledge the invaluable assistance of Miss Alice Jindra in planning and preparing the diets.

The use of dehydrated foods has been accompanied in some cases by a reduced acceptability. In some instances this may have been due to an unsatisfactory processing in the preparation of such products; in many more cases, it was probably related to an inadequate training or knowledge of the methods of preparation of such foods by the chefs. Methods which are satisfactory for the preparation of foods in the quantity needed by a small family may be unsatisfactory when used for the preparation of foods in the quantities required by the average army mess. However, processing difficulties have been largely overcome with increasing experience and the products now available are markedly superior to those available during the early days of the war. Greater experience in the preparation of dehydrated foods has lessened the failures encountered in the kitchen.

There has been some question about the satiety value of dehydrated foods as compared with the corresponding fresh foods. If satiety value is related to bulk, and a complete rehydration of such dried foods did not occur in their preparation, such assumptions might have some scientific basis. Other changes might also occur during processing which might affect the acceptability of the dehydrated food.

The present study was undertaken to determine whether dehydrated foods had the same nutritive value as fresh foods when tested by such methods as the determination of their digestibility, their ability to provide nitrogen equilibrium and to maintain body weight. The experiments were also designed to determine how the acceptability of dehydrated foods compares with that of fresh foods. It was hoped that the tests might give some information as to whether any alteration in water balance might accompany the use of such dried foods.

#### EXPERIMENTAL

##### *Subjects*

The studies were carried out on 8 male volunteers who were patients at the Rancho Los Amigos.<sup>4</sup> Some of them were

<sup>4</sup> This is one of the chronic units of the Los Angeles County Hospital System.

the same individuals who had served on a previous study on methionine (Johnson et al., '47). The men ranged from 32 to 56 years of age, from 123 to 179 lbs. in weight and from 66 to 75 inches in height. They were not suffering from any ailments which would be expected to alter their digestive processes or their nitrogen metabolism. The subjects were confined to one ward which was exclusively used for this test. A nurse or attendant was present at all times and during the day 2 nurses and an attendant were on duty. The subjects were allowed to leave the ward but only under supervision. Cigarettes, cigars and newspapers were furnished and the diets were more elaborate than the usual hospital fare. These factors combined with the personal attention made it possible for us to obtain cooperative subjects in spite of the monotony and prolonged confinement necessary for the experiments reported here.

### *Diets*

The diets were prepared in the diet kitchen at the Rancho Los Amigos, and the portions weighed out for the individual trays at the ward. The entire meal was eaten by the subject. The amount of water taken in was also controlled. During the last period of each test, the subjects were allowed to have the food *ad libitum* in additional portions of  $\frac{1}{4}$ ,  $\frac{1}{2}$ , or a portion equal to that usually taken with the stipulation that increased amounts of all components of the diet be consumed. In this way an attempt was made to obtain an index of the acceptability of the food.

Four menus were employed each of which provided approximately 12 gm of nitrogen and 2,000 cal. The order in which they were served was in some cases varied to prevent prediction of menu, but during each 8-day metabolic period, each menu was repeated twice. The components of the diets are given in table 1.

The composition of the 4 menus is indicated in table 2. Menu IV was served on Sunday since the cold meal in the evening made it the most practical for that day.

TABLE 1

*Diet components used in the studies on fresh and dehydrated foods.*

FRESH FOOD DIETS	DEHYDRATED FOOD DIETS
Canned beef, chicken, ham, lamb, pork or veal <sup>1</sup>	
Margarine	
Coffee	
Sugar	
Fresh Idaho potatoes <sup>2</sup>	Dehydrated diced potatoes <sup>2</sup>
Bread	Crackers
Apricots (canned, water-packed)	Dried apricots <sup>2</sup>
Peaches (canned, water-packed)	Dried peaches <sup>2</sup>
Whole milk	Spray-dried whole milk powder <sup>4</sup>
Fresh eggs	Dried egg white <sup>2</sup>
	Dried egg yolk <sup>2</sup>
Orange juice <sup>6</sup>	Orange juice concentrate <sup>7</sup>

<sup>1</sup> Specially prepared by arrangement with Dr. Victor Conquest, Armour and Co.<sup>2</sup> Obtained from Rogers Brothers, Idaho Falls, Idaho.<sup>3</sup> Obtained through the courtesy of Dr. Emil Mrak, University of California at Berkeley.<sup>4</sup> Challenge brand.<sup>5</sup> Obtained from the Seymour Packing Co., Topeka, Kansas.<sup>6</sup> Valencia oranges used.<sup>7</sup> Concentrate from Valencia oranges obtained from the California Fruit Growers Exchange, Ontario, California.*Analytical methods*

Nitrogen determinations were made on the urine, dried feces or aliquot of the mixed food sample by the macro Kjeldahl method. Urinary creatinine was determined colorimetrically using the Klett-Summerson colorimeter.

The analyses of the food samples were carried out on an aliquot of combined samples of extra meals weighed out at the regular mealtime. The samples from each day were thoroughly comminuted in a Waring blender and the contents were made up to approximately 3800 ml with measured amounts of water. This mixture was sufficiently liquid to be pipetted for nitrogen or water determinations. Fat was determined by the Soxhlet method on the dried residue.

TABLE 2

*The composition of the different menus employed.*

BREAKFAST		DINNER		SUPPER	
Food	Amt.	Food	Amt.	Food	Amt.
	gm		gm		gm
Menu I					
Scrambled egg	35	Lean roast beef	85	Potato soup	218
with diced ham	10	French fried		Margarine	7
Hashed brown		potatoes	125	Peaches	100
potatoes	205	Margarine	7		
Margarine	14	Apricots	100		
Sugar	35				
Menu II					
Egg omelet	54	Roast pork	50	Cold sliced beef	50
with diced ham	10	Buttered potatoes	206	Oven browned	
Diced browned		Margarine	14	mashed potatoes	138
potatoes	205	Apricot whip	87	Margarine	14
Margarine	14			Peaches	100
Sugar	35				
Menu III					
Scrambled eggs	45	Roast veal	40	Mashed potatoes	188
Fried potatoes	177	Baked potato		with diced	
Margarine	14	(no skin)	200	roast lamb	25
Sugar	30	Margarine	14	Margarine	7
		Baked custard	150	Peach in gelatin	153
Menu IV					
Potato cake	187	Roast chicken		Potato salad	200
Margarine	14	( $\frac{1}{2}$ light and		Margarine	9
Sugar	7	$\frac{1}{2}$ dark meat)	80	Apricots	100
		Mashed potatoes	188		
		Gravy	65		
		Margarine	9		
		Orange sherbert	166		

Additional foods served.

All meals — Bread 30 gm

Whole milk 169 gm (I and IV); 113 (II and III).

Breakfast — Black coffee, 180 gm

Strained orange juice, 183 gm (I, II, III); 198 gm (IV).

The diets in the 3 series of tests were calculated to contain approximately 12 gm of nitrogen. However, the protein levels were somewhat lower during the period when the dehydrated foods were fed. The variations in nitrogen determined on food samples in the several series are given in table 3.

TABLE 3  
*Nitrogen content of diets as determined by analysis.*

SERIES	MENU I	MENU II	MENU III	MENU IV
Series A (fresh) 5 samples each	12.88 (12.30-14.17)	12.99 (12.47-13.19)	11.32 (10.04-12.28)	13.61 (12.74-14.12)
Series B (dehydrated) <sup>1</sup> 8 samples each	11.36±0.20 (10.83-12.74)	10.51±0.37 ( 9.20-12.48)	10.70±0.23 ( 9.87-12.20)	12.88±0.16 (12.14-13.64)
Series C (fresh) <sup>1</sup> 10 samples each	13.28±0.26 (12.20-15.15)	12.99±0.17 (12.22-13.73)	12.54±0.15 (11.60-13.00)	13.69±0.18 (12.74-14.72)

The values in parentheses are the maximum and minimum values.

<sup>1</sup> Includes standard error of the mean calculated as follows  $\sqrt{\sum d^2/n}/\sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

### *Plan of the experiments*

The subjects were placed on the diets generally for 2 days as an orientation period before the collections of urine and feces were started. The urine collection was started at 7 A.M. prior to breakfast on each day. No markers were used in the separation of the feces and collections were begun simultaneously with those of the urine. It was considered that the 16- to 32-day periods employed were sufficient to minimize any errors to this procedure.

In the first series of tests carried out from December 3 to December 23, 1946, where fresh foods were employed (series A), only 3 periods were studied. The first 2 were each of 8 days duration when fixed amounts of the foods were ingested, while the third was an *ad libitum* period of 5 days. The second series of tests (B), which were made on dehydrated foods included three 8-day tests on the fixed diets followed by an 8-day *ad libitum* period (January 6-February 7, 1947). The third

series of tests on fresh foods consisted of four 8-day periods followed by an *ad libitum* period of 8 days (February 17–March 28, 1947).

## RESULTS

Table 4 records the data on changes in body weight as well as the figures on which protein and water exchanges are based. The results are the averages of 8 subjects for series A and B and for 7 for most of period C. One subject (no. 8) was transferred from the hospital so that he was no longer available for the third series of tests; another subject (no. 4) became ill during period IV of series C. However, since urinary creatinine was normal up to this period and no albumin or sugar appeared, the earlier data are included in the averages.

In spite of the fact that considerable losses in weight occurred during each period, the men were able to recover most of their weight during the *ad libitum* and the rest periods between series A and B and series B and C. The 2000 calorie level sufficed to maintain weight equilibrium with the shorter, lower weight subjects but was quite inadequate for 3 of the men who were tallest and heaviest.

The protein level was sufficiently generous so that the men were in positive nitrogen balance during all periods. The urinary nitrogen was somewhat lower during the periods when dehydrated foods were fed which is probably largely accounted for by the lower protein intake. Fecal nitrogen was quite constant throughout and gave identical averages in the periods with fresh and dehydrated diets. Although considerable fluctuations in urinary creatinine occurred in individual subjects from day to day, the average values for each period are quite constant for each subject. The variations may in part be related to variations in the intake of creatine since the different meats used certainly contained varying quantities of creatine. The average creatinine excretion per day was found to be identical on the fresh and dehydrated diets. We believe the creatinine fluctuations were not due to failure to secure quantitative collection of the urine.

TABLE 4

*The average body weight, nitrogen balance, water exchange and urinary creatinine of 8 male subjects who received diets of fresh or dehydrated foods.*

CATEGORY OF INTEREST	FRESH FOOD (A)			DEHYDRATED FOOD (B)				FRESH FOOD (C)						AVERAGE	
	I	II	Average	I	II	III	Average	I	II	III	IV	Average	A + C	B	
Number of subjects	8	8		8	8	8		7	7	7	6				
Body weight, lbs. <sup>1</sup>															
Start			155.8				153.8					154.0	154.9	153.8	
Change <sup>2</sup>			-2.45				-3.05					-3.90	-3.18	-3.05	
Nitrogen, gm/day															
Food	12.60	12.75	12.68	11.36	11.01	11.12	11.16	12.83	13.13	13.25	12.65	12.95	12.86	11.16	
			±0.25				±0.24					±0.15	±0.13	±0.24	
Urine	10.39	10.60	10.50	9.72	9.18	9.74	9.55	11.03	10.25	10.54	9.41	10.34	10.40	9.55	
			±0.27				±0.27					±0.36	±0.24	±0.27	
Feces	0.84	0.80	0.82	0.92	0.74	0.84	0.84	0.91	0.75	0.95	0.89	0.88	0.85	0.84	
			±0.06				±0.05					±0.06	±0.04	±0.05	
Balance	1.37	1.35	1.36	0.72	1.09	0.53	0.78	0.88	2.05	1.66	2.32	1.71	1.58	0.78	
			±0.26				±0.25					±0.36	±0.25	±0.25	
Creatinine, mg/day	1519	1531	1525	1487	1370	1630	1496	1707	1455	1420	1372	1492	1504	1496	
			±47				±53					±60	±42	±53	
Water, gm/day															
Food	1898	1961	1930	2017	2007	2022	2015	1930	1936	1903	1897	1916	1921	2015	
Urine	1346	1351	1349	1272	1196	1349	1273	1331	1302	1292	1352	1313	1329	1273	
Feces	40	38	39	45	37	44	42	45	41	53	43	45	43	42	
Balance	512	572	542	700	774	629	700	551	593	558	502	558	549	700	

<sup>1</sup> Values for 6 subjects only who completed all series of tests.

<sup>1</sup> Values for 6 subjects only who completed all series of tests.

<sup>2</sup> Value at 20 days for series A (including orientation period) and 21 days for series B and C.



The digestibility of the fat was calculated by the methods usually employed (Langworthy, '23). Correction was made for the metabolic fat according to the procedure of Langworthy and Holmes ('15). The pertinent data are summarized in table 5.

TABLE 5

Summary table of the fat ingested and the coefficient of digestibility on 8 male subjects.

SERIES OF TESTS	FAT IN GM PER 8-DAY PERIOD				COEFFICIENT OF DIGESTIBILITY <sup>2</sup>
	Eaten	Total in feces	Metabolic fat <sup>1</sup>	Feces fat (corrected)	
A. Fresh	(529.2) <sup>2</sup>	22.2	10.8	11.4	98.0 (96.0-99.5)
B. Dehydrated	555.8	18.6	10.5	8.1	98.5 (95.0-100.0)
C. Fresh	529.2	16.2	9.2	7.0	98.7 (96.3-100.0)

<sup>1</sup> Weight of dried feces  $\times$  0.0989.

<sup>2</sup> The values in parentheses indicate the range of the results obtained.

<sup>3</sup> Based on the values determined in series C where the same diet was employed.

The average wet and dry weights of the stools are included in table 6, along with the nitrogen analyses. These are presented as evidence that on uniform diets, considerable differences in bulk and dry weight of the stools obtain between different subjects. Moreover, there would appear to be a fair degree of uniformity in the nitrogen content of the stools in any one subject but marked variations in the nitrogen levels of the stools from different individuals.

The experiments where *ad libitum* periods were employed were designed to get objective information as to the acceptability of the fresh and dehydrated foods. These data are summarized in table 7. Because period A lasted only 5 days while B and C were for the standard 8-day interval, the response of the subject is calculated as the percentage additional rations consumed during the period. The previous effect of monotony is balanced since two and four 8-day periods preceded the *ad libitum* periods in series A and C, respec-

TABLE 6

*The stool bulk, dried weight and nitrogen content of the various subjects on diets of fresh or dehydrated foods.*

SUBJECT	WET WEIGHT GM/8 DAY PERIOD			DRIED WEIGHT GM/8 DAY PERIOD			NITROGEN CONTENT OF DRIED FEELS		
	Period A	Period B	Period C	Period A	Period B	Period C	Period A	Period B	Period C
1	321.2	378.2	329.1	87.6	96.5	77.2	7.6, 6.6	6.9, 6.4, 6.8	7.5, 7.2, 7.3, 6.9
2	605.2	562.4	475.4	139.0	134.6	111.3	7.6, 7.4	6.6, 6.8, 7.4	6.8, 7.1, 7.3, 7.2
3	596.3	657.1	538.8	116.2	116.7	89.3	5.7, 7.2	7.1, 7.3, 7.6	6.8, 7.3, 7.8, 7.4
4	221.4	223.2	287.2	80.2	82.6	103.1	4.8, 6.7	5.6, 5.5, 5.7	6.0, 5.8, 6.0, 5.8
5	370.0	418.9	420.2	86.4	100.6	96.0	5.7, 4.7	5.2, 4.7, 6.3	5.8, 5.7, 5.8, 5.8
6	379.5	524.2	535.3	79.1	113.1	106.4	6.0, 5.6	6.5, 6.1, 5.1	7.2, 6.7, 6.5, 6.2
7	565.9	547.2	550.6	136.1	116.9	120.2	7.6, 6.0	7.3, 7.2, 7.5	7.4, 7.7, 8.2, 7.6
8	282.5	221.0	....	94.2	80.4	....	5.9, 6.0	4.6, 5.2, 5.7	.....
9	....	....	407.1	....	....	117.0	.....	.....	6.2, 6.3, 6.4, 6.2
AV.	437.1	473.3	448.1	103.5	108.7	100.5			

TABLE 7

*The extra rations requested and eaten during the ad libitum period while receiving the fresh food diet (A or C) or the dehydrated food diet.*

SUBJECT NO.	PERCENTAGE EXTRA RATIONS EATEN			
	A	C	B	Average A + C
1	30	52	50	41
2	15	11	23	13
3 <sup>1</sup>	0	-25	-25	-12.5
4	0	0	4	0
5	40	24	64	32
6	40	37	35	38
7	0	0	0	0
8	20	..	38	20
9 <sup>2</sup>	..	44	..	44
Subjects Average 1, 2, 3, 4, 5, 6, 7, 8			31	21

<sup>1</sup> Subject had gained weight and preferred to eat less. Since an opportunity for a reduced intake was not given for period A, these results are not included in average.

<sup>2</sup> Omitted from the average since no comparison was available on diets with dehydrated foods.

tively, compared with three 8-day periods preceding the *ad libitum* period in series B.

#### DISCUSSION

The present experiments indicate that food dishes prepared from dehydrated products may have as high a degree of acceptability as those made from fresh foods. This does not entirely agree with the experience of the army where difficulties in some cases occurred when such processed foods were used.

There are 2 possible explanations for the above discrepancies. In the first place the quality of the dehydrated foods now available and which we used must in general be higher than in the early war years as a result of the vast amount of experience in such processing. Moreover, the products we employed were quite fresh and in no case showed any deterioration. Likewise, methods of preparation which proved to be entirely adequate for limited quantities of material in some

cases would be unsatisfactory without modification for the amounts needed for an army mess. In testing out recipes for dehydrated potatoes in the present tests, excellent results were obtained in pilot experiments; however, when the same proportions and methods of preparation were employed for the larger amounts required for the diets, failure resulted. The procedures had to be entirely changed before the products prepared on the larger scale had an edibility which compared favorably with those cooked in the pilot experiments.

One criticism that has been leveled against dehydrated foods is the lack of satiety value. This criticism was not borne out in the present experiments although we have no objective tests to report on it. However, the remarks of the subjects and the answers to questions indicated that the diets prepared from dehydrated foods were equally satisfying to them as those prepared from the fresh foods. Although such data are not quantitative, there is evidence from more recent tests that we might have expected to experience an immediate response from the men had the diet not been satisfactory from the standpoint of satiety. When the potatoes were replaced by bread but the same number of calories administered, the men complained repeatedly over the several weeks that the experiments were characterized by continued hunger (Johnson and Deuel, '47).

The digestibility of the diets shows no differences ascribable to the type of food used. The nitrogen loss in the feces which is generally considered to be largely present in the bacteria was identical in the 2 types of diets, being 0.84 and 0.83 gm, respectively, when the fresh or dehydrated diets were fed.

The digestibility of the fat in the 3 series of tests was found to be especially high. The average in both series was 98% or higher where an average of over 60 to 70 gm of fat was consumed daily. Since the fats were primarily made up of margarine fat, cream and egg yolk fat with minimal amounts of the meat fats, the high digestibility is not unexpected. The values previously reported for the digestibility of egg yolk fat in man are 93.8 (Langworthy and Holmes, '17),

for cream, 96.9 (Langworthy and Holmes, '17) and for margarine fat, 97.2 (Deuel, '46). It is therefore evident that as satisfactory fat utilization obtains with the dehydrated diets as with the fresh ones.

Although no determinations were made of the digestibility of other foodstuffs, the uniformity of the dry weight of the feces in the 3 series of tests would seem to offer evidence of the uniformity of assimilation of the other foodstuffs on diets prepared from dehydrated and fresh foods.

Another indication of the relative nutritive value of the 2 types of diets may be gleaned from the urinary nitrogen levels. The average value of 9.54 gm daily for the samples from the subjects during period B is considerably lower than the average of 10.34 gm per day for the subjects who received the fresh food diet (periods A and C). However, this slightly lower figure in the former case must be the result of the lowered consumption of protein when eating the dehydrated diets. Although the positive nitrogen balance is considerably higher for the subjects receiving the fresh food diet as contrasted with the period when they received the dehydrated diets, it is believed that this is to be attributed largely to the higher level of protein fed.

One of the best indices of nutritive value of a food is its ability to maintain body weight in the adult. Since the food intakes in most cases were too low to allow a caloric equilibrium a gradual decline in weight occurred during the experimental periods. The average loss of weight after 21 days on the dehydrated foods diet (3.05 lbs.) was certainly not greater than that after an equal interval on the fresh food diets (3.18 lbs.).

Although the water balance does not include any figures for the water obtained by the combustion of foods nor does it allow for the loss of water by "insensible perspiration," there is no evidence of an upset in the pathways of excretion of the water. The slightly greater positive water balance in the dehydrated series (700 vs. 548 gm) may be explained on a somewhat higher water intake (94 gm) and the lower excre-

tion in the urine in that series. The lower urine volume might be explained because of the lessened diuresis which resulted from the lower urea excretion.

Stool bulk as well as the dried weight of the feces were approximately the same in all 3 series of tests. Marked variations in the quantities excreted by different subjects were noted. Three subjects (2, 3, 7) consistently excreted between 475 and 600 gm of stools during the 8-day interval while 2 other subjects (4, 8) had a maximum stool weight under 300 gm. Similar variations in dry weight are to be noted. In general, the samples which weighed the least were those containing the lowest percentage of nitrogen while the high values in nitrogen were generally associated with a large production of feces.

It would also appear from the data presented here, that the same subject on a uniform diet excretes a stool with fairly uniform nitrogen content. The maximum value for nitrogen obtained in subjects 4, 5 and 8 was 6.0% while the minimum nitrogen level in subjects 1, 3, 7 and 9 was 6.0%.

One must conclude that by the methods available for evaluation of nutritive value in human subjects, namely digestibility, urinary nitrogen, stool bulk, weight retention and acceptability, dehydrated foods compare satisfactorily with the comparable fresh foods.

#### SUMMARY

1. Prolonged feeding tests were made on 8 male subjects with fresh food diets and comparable diets made from dehydrated foods.

2. Protein and fat were equally well digested in the diets made from dehydrated and fresh foods. Although the positive nitrogen balance was somewhat higher on the fresh food diets, it is probably due to the somewhat higher level of protein in those tests. Considerably lower urinary nitrogen values were obtained on the diets of the dehydrated foods than on those made from fresh food. Losses in body weight on the 2000 calorie level were similar on the 2 types of diets.

3. The average loss of nitrogen in the feces was identical on the diets of fresh and dehydrated foods.

4. No alterations in the pathway for the metabolism of water were found.

5. The acceptability of the dehydrated food diet as determined by *ad libitum* tests was equally good in the fresh and dehydrated foods.

6. Stool bulk showed wide variations between the different subjects but was relatively constant on any one subject irrespective of the diet.

7. Considerable uniformity was noted in the nitrogen percentage in the dried stools from any one subject on a fairly constant diet although marked variations were noted in the values obtained on the stools of the different subjects.

8. A slightly positive nitrogen retention was observed in subjects receiving the diets prepared from the fresh or dehydrated foods in spite of the fact that they were losing body weight because of low caloric intake.

9. It is concluded that the diets made from dehydrated foods may be equally as nutritious as those prepared from fresh foods.

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# THE THIAMINE AND RIBOFLAVIN CONTENT OF WHOLE WHEAT, NONENRICHED AND EN- RICHED FLOURS AND OF BREADS MADE THEREFROM

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TWO FIGURES

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The cereals have long been regarded as very important constituents of the human diet, especially that of low-income peoples. In many countries, the process of preparing wheat for human consumption has changed greatly during the past century. With these changes in processing or milling practices, there has arisen some concern as to the probable effects of modern milling methods on the nutritive value of wheat products. Of special concern has been the effect of the milling process, in the production of white flour, in removing certain portions of the wheat berry which are high in essential vitamins and minerals. The nutritional virtues of whole wheat flour and so-called dark breads, as contrasted to those of white flour and white bread, have been proclaimed by numerous adherents. Likewise the superior keeping qualities, the greater public acceptability, more desirable baking properties, and other characteristics of white flour have been similarly propounded. Suggestions have been advanced as to how the nutritive value of white flours may be improved by incor-

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porating in them, or in the breads made therefrom, certain vitamins, minerals and protein supplements.<sup>2</sup>

Among the more recent publications of experimental work dealing with various comparisons of whole wheat flour and enriched white flour is that of Westerman and Bayfield ('45) who studied the B-complex vitamin content when these products constituted 30 to 50% of the diet of the test animals. These studies included flours enriched in accordance with the previous standard as well as in accordance with the present standard, and it was found that whole wheat was a better source of the B-complex vitamins than was either the "Morris type" flour or the white flour which had been enriched on the basis of the previous standard when these products constituted 30 to 50% of the diet. At the 30% level of feeding, the whole wheat was found to be slightly better than white flour that had been enriched in accordance with the present standard. Whole wheat and enriched flour were found to promote the same rate of growth when fed at the 40% level, while at the 50% level enriched flour promoted a faster rate of growth than did whole wheat.

The authors failed to offer an explanation as to why whole wheat, when fed at the 30% level, supported better growth in rats than did the white flour which had been enriched according to the present formula, while at the 50% level of feeding the enriched flour produced greater gains in body weight than did the whole wheat. From the above report it would appear that either the flour which had been enriched according to the present standard contained dietary essentials not present in the wheat berry or that the natural vitamins (thiamine and riboflavin), although present in the whole wheat in somewhat lower concentration, were utilized more efficiently than were the synthetic vitamins added in the enrichment process, especially when the wheat and the flour

<sup>2</sup> A literature résumé (Monograph) by Dunlap "White versus Brown Flour" was published in 1945. The enrichment of white flour and white bread in the United States began in 1941, and an excellent review may be found in the National Research Council publication, Bulletin no. 10, November, 1944, "Enrichment of Flour and Bread—A History of the Movement."

were incorporated in the diet at or near the critical level (30%).

From reported researches it is difficult to determine the validity of many of the conclusions that have been drawn regarding the relative nutritional merits of whole wheat, white flour and enriched white flour. This is partly due to the fact that the origin of the wheat and of the flours has not been stated in some instances, and where this information has been given it was obvious that frequently the flours under investigation had not been made from the wheat to which their nutritive value was compared.

It appears that the most reliable basis on which to compare the relative nutritive values of whole wheat and wheat flours would be to have all products under investigation come from the same natural source; in this instance, from the same wheat. It also appears that a study of this particular type, especially when wheat and flours of the same origin were involved, would not be complete unless the investigation also included similar studies with breads made from the wheat and the flours in question.

The studies reported at this time were conducted with the view of ascertaining the effectiveness of the present enrichment practice in supplementing the thiamine and the riboflavin content of white flour and also how much of these vitamins is retained by breads made from the wheat and from these flours when the breads are made in accordance with standard baking procedures. These studies have involved only the type of enriched flour produced under the present enrichment formula which has been in effect since October 1, 1943.

#### EXPERIMENTAL

##### *Materials tested*

As basic materials for use in the investigation, 200 lbs. of each, whole wheat, nonenriched flour and enriched flour,

all having a common origin, were obtained.<sup>3</sup> The flour had been produced from the wheat through the usual commercial milling practice and the enriching of the flour had been carried out on a commercial scale. The wheat contained 2.32% nitrogen while the flours contained 2.08% nitrogen (moisture-free basis). The flour was of 62% extraction. The whole wheat, the nonenriched white flour, and the enriched white flour were each thoroughly mixed, transferred to air tight containers and stored at 60°F. until made into bread or until incorporated into the various experimental diets. As it was used, the whole wheat was ground to a high degree of fineness by means of a power driven mill.

Inasmuch as there have been arguments presented in favor of dark bread and of milk bread just as there are arguments in favor of whole wheat bread and of enriched bread, it was decided to include both dark bread and milk bread in the present studies. The dark bread was made from a mixture of flours consisting of 30% whole wheat flour and 70% non-enriched white flour. The milk bread was made by incorporating 1.5 lbs. of dry milk solids (non-fat) in a bread mix containing 25 lbs. of the nonenriched flour. The compositions of the 5 bread mixes are given in table 1. Sufficient distilled water was added to the mixed ingredients to form a heavy dough. The doughs were made into approximately pound loaves and baked in the usual manner for 25 minutes at 450°F. This was carried out in a local bakery operating under commercial conditions.

The baked breads were removed from the oven, taken immediately to the laboratory and weighed. The breads were then crumbled and subjected to preliminary dehydration by being exposed to air at room temperature. The final dehydration was carried out by allowing the breads to remain overnight in a large hot air oven operating at 160–162°F. The dried breads were ground, placed in air-tight containers

<sup>3</sup>The authors wish to acknowledge the cooperation of General Mills, Inc., Minneapolis, Minnesota, and Merck and Co., Inc., Rahway, New Jersey, in making these studies possible.

TABLE I  
*Composition of breads.*

COMPONENTS	WHOLE WHEAT BREAD	NONENRICHED BREAD	ENRICHED BREAD	DARK BREAD	MILK BREAD
	lbs.	lbs.	lbs.	lbs.	lbs.
Flour	25.0	25.0	25.0	25.0	25.0
Sugar	1.0	1.0	1.0	1.0	1.0
Salt	0.5	0.5	0.5	0.5	0.5
Shortening (Crisco)	0.5	0.5	0.5	0.5	0.5
Yeast food	0.1	0.1	0.1	0.1	0.1
Milk powder (skim)					1.5
Malt syrup	2.0 oz.	2.0 oz.	2.0 oz.	2.0 oz.	2.0 oz.

Water was added to the above ingredients in a sufficient amount to form a heavy dough. The dough was made into approximately pound loaves and baked in the usual manner for 25 minutes at 450°F. This was carried out through the co-operation of a local bakery operating under commercial baking procedure.

and stored at approximately 36°F. until incorporated in the various diets. The changes in the moisture content of the various breads during baking and during subsequent drying are indicated by the data presented in table 2.

### *Plan of testing*

Since the preponderance of evidence indicated that the pre-war American diet contained approximately 30% of its calories in the form of cereals, this percentage offered the basis for incorporating the ground whole wheat, the nonenriched flour, the enriched flour and the breads made from these products in the various diets in order to compare their relative thiamine and riboflavin content. Hence the ground whole wheat and the white flours were incorporated in the diets at a level equivalent to 30% of the original flour. This was done by taking into consideration weight changes due to added ingredients other than flour and to moisture additions and losses. The ground wheat, flours and breads replaced an equal weight of sucrose from the basal diet. All diets were made up at frequent intervals and were stored at 36°F. until used. The percentage composition of the 9 basal diets employed as well as the amounts of B-vitamin supplements used are

TABLE 2  
*Changes in the moisture content of the breads during baking and subsequent drying.*

	WHOLE WHEAT BREAD	NON-ENRICHED BREAD	ENRICHED BREAD	PAKE BREAD	MILK BREAD
Amount of flour used, gm	11,360	11,360	11,360	11,360	11,360
Amount of moisture in flour, %	10.52	12.07	11.34	10.95	12.07
Total weight of ingredients, gm	12,305	12,305	12,305	12,305	12,980
Number of loaves produced	40	38	39	40	40
Weight of fresh bread, gm	17,000	15,900	15,925	16,154	16,970
Water uptake, gm	4,695	3,595	3,620	3,949	3,990
Weight of dried bread, gm	11,600	11,180	11,100	11,120	11,825
Residual moisture in dried bread, %	1.85	2.91	1.66	2.45	2.55
Water uptake minus water loss, gm	--765	--1,125	--1,205	--1,085	--1,155
Bread/flour ratio	1.02	0.98	0.98	0.98	1.14

given in table 3. The vitamin supplements other than thiamine, riboflavin and niacin were incorporated directly in the diets, while thiamine, riboflavin and niacin were fed separately. The thiamine, riboflavin and niacin contents of the flours, breads and diets were determined at regular intervals during the course of the feeding tests. In carrying out these assays, thiamine was determined in the enzyme digested extracts by the method of Conner and Straub ('41), riboflavin by the Snell and Strong microbiological method ('39) except

TABLE 3  
*Percentage composition of the basal diets employed.*

DIET NUMBER	1	2	3	4	5	6	7	8	9
<i>Ingredients</i>									
Purified casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Salt mixture	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Hydrogenated cotton-seed oil	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Cod liver oil	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Cell-U-Flour	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Sucrose	64.0	34.0	34.0	34.0	34.0±	34.0±	34.0±	34.0±	34.0±
Whole wheat flour <sup>2</sup>	30.0								
Nonenriched flour <sup>2</sup>			30.0						
Enriched flour <sup>2</sup>				30.0					
Whole wheat bread <sup>1,2</sup>					30.0±				
Nonenriched bread <sup>1,2</sup>						30.0±			
Enriched bread <sup>1,2</sup>							30.0±		
Dark bread <sup>1,2,3</sup>								30.0±	
Milk solids bread <sup>1,2,3</sup>									30.0±
Other B-vitamins <sup>4</sup>	+	+	+	+	+	+	+	+	+

<sup>1</sup> All breads were included in the diets on the basis of content of original flour (30% of flour in the diet), the differences in weight between bread and flour being compensated for by adjusting percentage of sucrose.

<sup>2</sup> Made from 30 parts of whole wheat flour and 70 parts nonenriched flour.

<sup>3</sup> Contained 6 parts of skim milk solids.

<sup>4</sup> Supplemented with 200 µg pyridoxine hydrochloride, 800 µg Ca pantothenate, 100 mg choline chloride, 4.0 mg *p*-aminobenzoic acid and 4.0 mg inositol per 100 gm of diet.

<sup>5</sup> The thiamine, riboflavin and niacin contents of these flours and breads (in mg %), in the order listed, were as follows: thiamine—0.43, 0.06, 0.46, 0.39, 0.07, 0.43, 0.12 and 0.03; riboflavin—0.09, 0.03, 0.29, 0.08, 0.02, 0.24, 0.04 and 0.14; niacin—3.52, 0.69, 3.97, 3.53, 0.66, 3.76, 1.39 and 1.09, respectively.

that the concentrations of glucose and of sodium acetate in the finally diluted medium were increased to 2.0 and 2.6%, respectively, as suggested by Stokes and Martin ('43), and niacin by the Krehl, Strong and Elvehjem ('43) modification of the Snell and Wright method ('41).

Diet 1 served as the control since it was essentially free of thiamine, riboflavin and niacin but contained all of the other dietary ingredients known to be required by the growing rat. Diets 2, 3 and 4 contained the flours while diets 5, 6, 7, 8 and 9 contained the breads made from these flours.

TABLE 4  
*Analysis of diets.*

DIET NO.	MOISTURE	ASH	ETHER EXTRACT	CRUDE FIBER	PROTEIN (N x 6.25)	N.F.E. <sup>1</sup>	VITAMIN CONTENT		
							Thiamine	Riboflavin	Niacin
	%	%	%	%	%	%	mg %	mg %	mg %
1	1.46	3.25	9.94	1.25	16.88	67.22	0.00	0.02	0.03
2	4.95	3.74	10.96	2.09	21.56	56.80	.14	.05	1.11
3	4.31	3.33	10.14	1.26	20.93	60.03	.02	.03	.26
4	4.83	3.26	10.59	1.20	21.06	59.06	.14	.12	1.36
5	2.21	3.86	12.51	2.00	21.44	57.98	.11	.04	1.20
6	3.04	3.46	11.17	1.19	21.50	59.64	.02	.02	.19
7	2.53	3.56	11.68	1.28	21.06	59.89	.13	.10	1.16
8	2.19	3.99	11.42	1.84	20.88	59.68	.04	.03	.45
9	2.84	4.71	11.25	1.37	21.25	59.58	.03	.06	.33

<sup>1</sup> Nitrogen-free extract.

The compositions of the 9 diets as well as their average thiamine, riboflavin and niacin content, as revealed by analyses, are given in table 4. Each of the 9 diets was fed unsupplemented, supplemented with riboflavin and niacin, with thiamine and niacin, and with thiamine, riboflavin and niacin. While no beneficial effects were expected as the result of the niacin supplementation, this vitamin was included among the supplements so as to approach conditions comparable to those found in whole wheat flour or in enriched flour. In carrying out the supplementation, the thiamine, riboflavin and niacin were dissolved separately in a 10-90%



alcohol-water solution and were fed daily in the following amounts: 13.2  $\mu$ g, 8.0  $\mu$ g, and 105.7  $\mu$ g, respectively. These dosages were calculated to be the equivalent of the intake of these vitamins when the test animals were subsisting on the enriched flour diet and were consuming, on an average, 10 gm of the diet daily.

### *Test animals*

All test animals employed in the investigation were taken from our breeding colony. A total of 38 experimental groups comprising 450 young rats were used. Thirty-seven of the experimental groups contained 12 animals each (6 males and 6 females), while the remaining group consisted of only 6 animals (3 males and 3 females). The animals of this latter group [group 1 (2d)] received the control diet supplemented by a double portion of the 3 vitamins.

The young rats were taken from the breeding colony when they were 21–23 days of age, at which time their weight ranged between 40 and 45 gm (average 43.3 gm). The animals were placed directly in individual all-metal cages which were provided with raised screen floors (2 meshes per inch) and were subjected to a 2-week adaptation period. During this period each animal received distilled water and a liberal allowance of the control diet (diet 1) without the supplement of thiamine, riboflavin and niacin. At the end of the 2-week adaptation period, the animals were distributed into experimental groups while taking into consideration the usual precautions to distribute litters, sexes and body weights uniformly throughout the various groups. Since the total number of test animals involved was considerable and could not be readily handled at one time owing to other phases of research in progress, the feeding tests were divided into 2 comparable series, and in consequence, only one-half of the animals comprising each experimental group was placed on experiment at one time. At the conclusion of the first series of tests, a second and similar series of feeding tests was conducted.

In all of these tests the diet was fed *ad libitum* and clean distilled water was kept before the animals at all times. The thiamine, riboflavin and niacin supplements were fed in supplement cups independent of the test diet. Daily observations were made for the purpose of detecting early indications of thiamine and riboflavin deficiencies. A careful record was made of individual food consumption and of changes in body weight. All animals remained on experiment for 8 weeks (exclusive of the 2-week adaptation period) unless death intervened.

#### DATA

The data obtained in the 2 series of feeding tests have been gathered together and reduced to tabular and graphic form and are presented in tables 1 to 4 and in figures 1 and 2.

#### DISCUSSION

##### *Response of rats receiving the control diet*

During the first 7 days of the 14-day adaptation period, the test animals consumed, on an average, 32 gm of the thiamine-riboflavin-niacin free diet (diet 1, table 3) and made favorable growth responses (average of 7.3 gm). However, the response during the last 7 days of the 14-day period was remarkably different. The overall average food consumption was only 18 gm per animal and more than 80% of the rats either failed to gain in weight or lost weight. The average net weight change for the 450 test animals during the 7-day period was a loss in weight of 1.5 gm per rat. While cessation of growth at this time was apparently due to thiamine deficiency, no outward appearance of such a deficiency was evident other than loss of appetite and cessation of growth. At this time the test animals were distributed into the several experimental groups.

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Fig. 1 The average food consumption and the average growth response of those groups of rats which received the control diet (diet 1) and the flour-containing diets (diet 2—whole wheat flour; diet 3—nonenriched flour; and diet 4—enriched flour), unsupplemented and supplemented with riboflavin and niacin, with thiamine and niacin, and with thiamine, riboflavin and niacin.

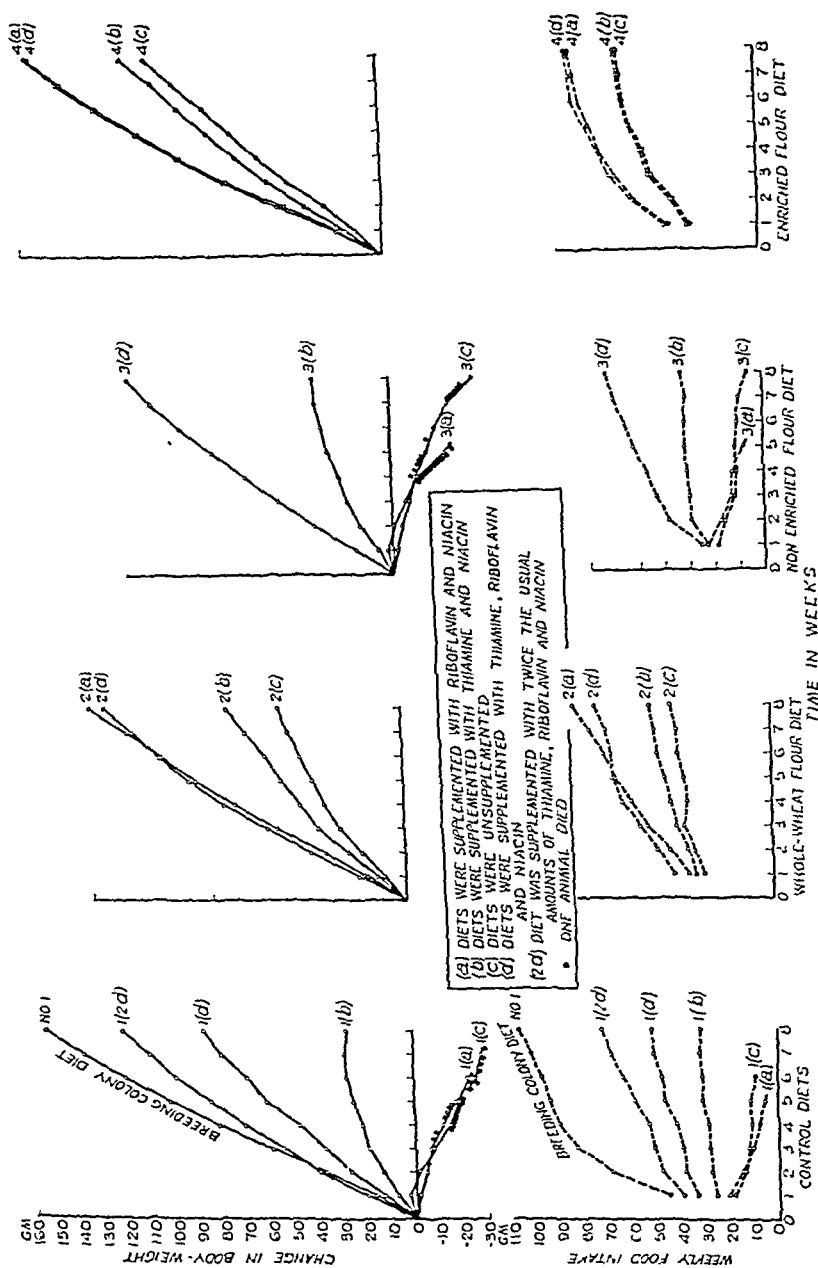


Figure 1

Those animals which were continued on the basal diet and which received a supplement of riboflavin and niacin [group 1 (a), fig. 1] made a very slight growth response during the first week of supplement feeding. However, the response was only temporary in that the animals constituting the group again declined in weight and all succumbed before the end of the sixth week of the test period. These results served to confirm our expectation that the basal diet was reasonably deficient in thiamine. When a similar group of animals received the thiamine-niacin supplement [group 1 (b)], a slow but consistent growth response resulted which continued throughout the first 6 weeks of the test period. This indicated that the basal diet either contained measurable amounts of riboflavin or that the body stores of riboflavin had not been completely eliminated during the 2-week adaptation period. Since the test animals did not continue to grow during the last 2 weeks of the test period, the authors are inclined to believe that the prolonged growth was due to the latter condition.

The group of rats which remained on the unsupplemented control diet [group 1 (c)] continued to lose weight, and all were dead by the end of the seventh week of the test period. However, since the animals comprising this group lived somewhat longer, on an average, than did a comparable group of animals subsisting on the same diet but receiving the riboflavin-niacin supplement, it appears that the feeding of additional amounts of 1 or more vitamins may accentuate the deficiency symptoms arising from lack of another vitamin. Similar observations have been made frequently during the past several years in connection with other types of vitamin studies (Guerrant et al., '37).

When the control diet was supplemented with thiamine, riboflavin and niacin [group 1 (d)], an immediate growth response resulted which continued throughout the test period. The animals of this group gained an average of 86 gm during the 8-week period and appeared to be in a reasonably good state of nutrition at the completion of the feeding test. Since

Westerman and Bayfield ('45) had found that flour enriched in accordance with the present formula, when fed at the 50% level, did not produce optimal growth in young rats, the question remained as to whether the suboptimal growth was due to further deficiencies of thiamine and riboflavin or to other characteristics of the experimental diet. In consequence, the second group of young rats (only 6 animals) receiving the control diet was given a double allowance of the thiamine-riboflavin-niacin supplement. While these animals [group 1 (2d)] made increased growth responses (15 gm per week) as the result of receiving the additional allowance of vitamins, they did not attain the growth rate of young rats subsisting on our breeding colony diet (group 1) during the same period (19.3 gm per week). Whether further supplementing of the diet with thiamine and riboflavin would have brought about additional improvements in the growth rate cannot be stated inasmuch as additional tests were not made. However, the data obtained are sufficient to show that the primary deficiencies in the control diet used in the present studies, insofar as limiting growth in young rats, were those of thiamine and riboflavin.

*Responses obtained with the flour-containing diets  
(diets 2, 3 and 4 of table 3)*

When 30% of freshly ground wheat was incorporated in the control diet in place of a similar weight of sucrose (diet 2), definite improvement in the growth rate resulted [diet 2 (c), fig. 1]. All animals comprising the experimental group responded immediately to the change in diet and grew continually during the 8-week test period. While the growth rate was not optimal (average of 6.4 gm per week) the results demonstrated that the ground wheat used in these studies, when incorporated in the ration at the 30% level, furnished sufficient thiamine and riboflavin to sustain life and to support a slow but consistent rate of growth. When this latter diet was supplemented with riboflavin and niacin [group 2 (a)] good growth resulted (129 gm in 8 weeks) and the animals

appeared to be in excellent condition at the end of the test period. Although the thiamine-niacin supplement proved less effective in promoting growth in young rats [group 2 (b)] subsisting on this diet than did the riboflavin-niacin supplement, the animals did respond to the former supplement and grew at an average rate of 9.0 gm per week in comparison with 6.4 gm gains made by comparable animals receiving the unsupplemented diet. This would suggest that 30% of ground whole wheat, when used to replace an equal weight of sucrose from the control diet, did not furnish adequate thiamine for optimal growth and that additional growth could be brought about by feeding a thiamine supplement. However, when the whole wheat flour diet was supplemented with thiamine, riboflavin and niacin [group 2 (d)] no greater increases in body weight were obtained than those observed without the thiamine supplement. Under the conditions of these experiments the feeding of the thiamine-riboflavin-niacin supplements did result in a slightly improved growth rate during the first 5 weeks of the test period, but this advantage disappeared during the next few weeks. While this latter observation remains unexplained, the data obtained in this phase of the investigation confirm previous reported observations that riboflavin deficiency is of primary consideration where whole wheat and wheat products are concerned.

Growth studies with diets containing 30% of nonenriched flour, unsupplemented and supplemented with riboflavin and niacin, with thiamine and niacin, and with thiamine, riboflavin and niacin [groups 3 (a, b, c and d)] yielded results which proved to be remarkably similar to those obtained under comparable conditions while using the control diet. However, there was evidence that the diet carried somewhat more thiamine and definitely more riboflavin as the result of including the 30% of nonenriched flour. Here again the rats receiving the riboflavin supplement, in the absence of thiamine, died at an earlier date than did comparable animals which did not receive the riboflavin.

When 30% of enriched flour replaced an equal weight of sucrose from the control diet, satisfactory growth responses were obtained both with and without additional vitamin supplements [groups 4 (a, b, c and d)]. In fact more favorable growth was obtained in all 4 instances than was obtained under comparable conditions of experimentation in which the diet contained 30% of ground whole wheat. Here again the thiamine supplement proved less effective in promoting additional growth than did riboflavin. In regard to the data presented in figure 1, it is of interest to note the consistent relationship between food intake and growth.

#### *Responses obtained with bread-containing diets*

Growth studies with diets containing the breads in amounts equivalent to 30% ground wheat or 30% of the flours from which the breads were made (diets 5, 6, 7, 8 and 9, table 3 and fig. 2) yielded data which were remarkably similar to those obtained with the previously described flour-containing diets (fig. 1). In most instances, growth was slightly greater on the bread diets than on the corresponding flour-containing diets. Mortality was also lower among the thiamine-deficient bread-fed animals. These apparent differences in nutritive value of the flour and of the bread-containing diets may, perhaps, be attributed to 2 possible factors, namely, to the more readily accepted bread diets and to the probable addition of vitamins or other growth stimulating nutrients in connection with the making of the breads. In regard to the former possibility, it can be definitely stated that in 9 of the 12 comparisons, the bread-fed animals consumed on an average more of the diet than did the flour-fed animals, while in 2 comparisons the amount of diets consumed by the 2 groups of animals was the same, and in one comparison the bread-fed rats consumed slightly less food than did the flour-fed animals. As to the latter possibility, it is recognized that traces of thiamine and riboflavin may have been added to the bread-containing diets by incorporating small amounts of yeast food,

malt syrup and live yeast culture in the bread mix (table 1). However, periodic analyses of breads and diets (tables 3 and 4) failed to show that the breads contained any more thiamine or riboflavin than did the flours from which they were made. In fact the analytical data indicate that the breads had sustained a slight loss in these vitamins apparently as the result of baking, drying and other preparatory measures preliminary to feeding.

Studies with the whole wheat bread diet [groups 5 (a, b, c and d)] reveal that sufficient thiamine was present to support satisfactory growth and that only a slight improvement in growth resulted when additional thiamine was fed. On the other hand, when the diet was supplemented with riboflavin, with and without additional thiamine, an increased rate of growth resulted, just as it had with the corresponding flour-containing diet. Although the animals which received a diet containing the bread made from the nonenriched flour [groups 6 (a, b, c and d)] grew at a slightly faster rate than did those animals receiving the flour diet, the responses obtained through feeding of the 2 diets, unsupplemented and supplemented, were remarkably similar.

The groups of rats receiving the diet containing the bread made from the enriched flour, without and with vitamin supplementation [groups 7 (a, b, c and d)], grew at approximately the same rate as did the animals receiving the corresponding flour diet. Here again the data clearly show that the amount of riboflavin added through enrichment was not adequate for optimal growth when the enriched flour or the bread made therefrom was incorporated in the diet at the 30% level, although the amount of this vitamin present in the enriched flour was somewhat greater than that found in the whole wheat or the whole wheat bread.

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Fig. 2 The average food consumption and the average growth response of groups of rats which received the bread-containing diets (diet 5—whole wheat bread; diet 6—nonenriched bread; diet 7—enriched bread; diet 8—dark bread; and diet 9—milk bread), unsupplemented and supplemented with riboflavin and niacin, with thiamine and niacin, and with thiamine, riboflavin and niacin.



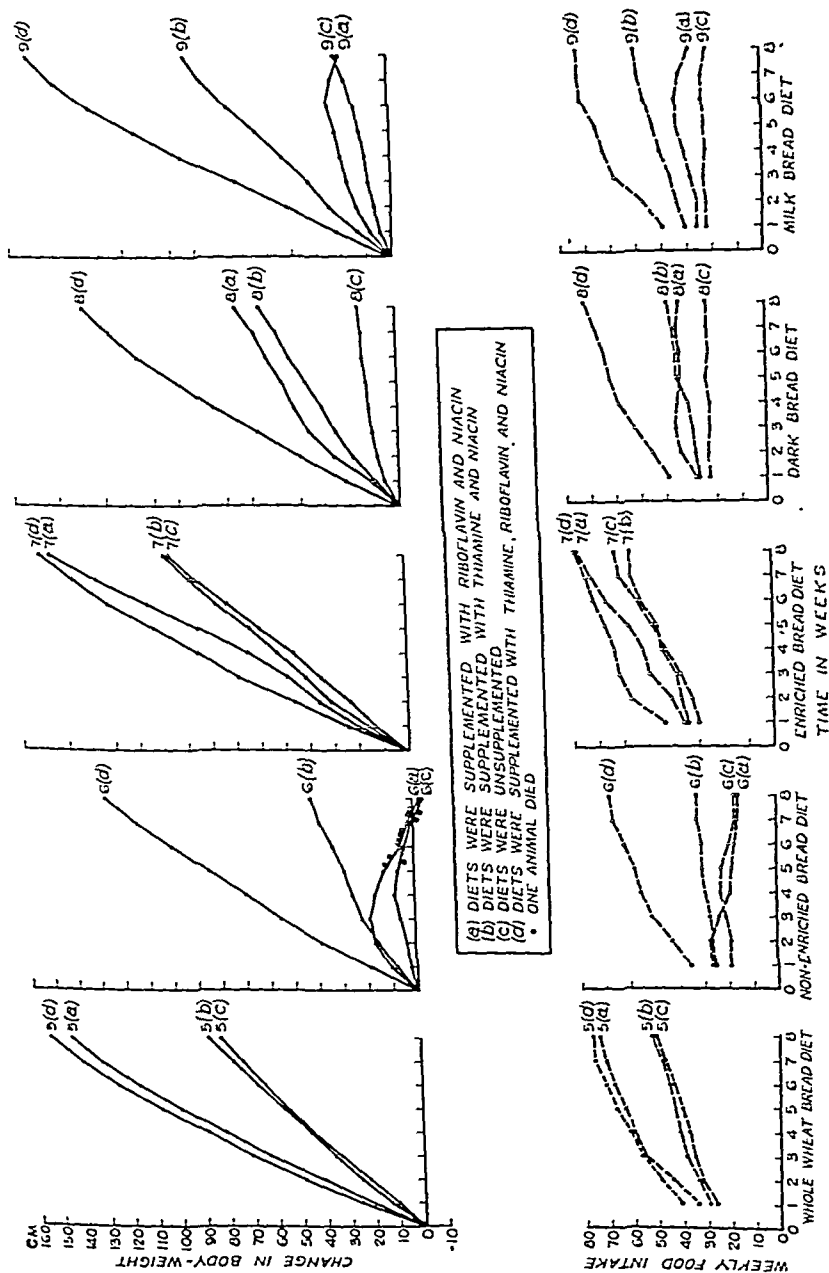


Figure 2

Studies with diets containing the dark bread (30% ground whole wheat and 70% of nonenriched flour) show that while the incorporation of a relatively small amount of finely ground whole wheat in the bread did result in improved growth and survival, the diet containing this bread was definitely deficient in both thiamine and riboflavin [groups 8 (a, b, c and d)]. The incorporation of 6% of skim milk powder in a bread made of the nonenriched flour resulted in the product having increased growth-stimulating and life-sustaining capacity [group 9 (c) vs. group 6 (c)], but it was inferior to enriched white bread in these respects [group 7 (c)]. From the standpoint of vitamin supplementation, it appears that the milk powder did add an appreciable amount of riboflavin but very little thiamine. This is borne out by the fact that additional riboflavin supplementation, without additional thiamine, did not improve the growth rate [group 9 (a)], and that thiamine supplementation in the presence of existing riboflavin resulted in a marked improvement in growth [group 9 (b)]. When this diet was supplemented by thiamine, riboflavin and niacin [group 9 (d)], excellent growth resulted. While these results do support existing contentions that the addition of skim milk or skim milk solids, in the making of unenriched white bread, enhances the nutritive value of the bread, the inclusion of 6% of skim milk solids, in this instance, did not add sufficient thiamine or riboflavin to make the bread equivalent with respect to these vitamins to the bread made from ground whole wheat or from enriched flour. Here again the close relationship between food consumption and growth is readily observed (fig. 2).

Periodic thiamine, riboflavin and niacin assays made during the course of the studies indicated that the thiamine, riboflavin and niacin content of wheat, flours, breads and diets remained constant. Although the experimental conditions were somewhat dissimilar, the results obtained in these studies substantiate in part those recently reported by Westerman and Hall ('47) in which it was shown that further supplementation of enriched flour with B-complex vitamins improved

the nutritive value of the product. In the present studies this was found to be true of riboflavin supplementation. Furthermore, riboflavin supplementation of the whole wheat diets was more effective in stimulating additional growth in young rats than when this vitamin was employed as a supplement to the enriched flour diet.

While the present investigation has been concerned primarily with the relative thiamine and riboflavin content of whole wheat, nonenriched flour and enriched flour, the authors are aware that whole wheat may contain desirable nutrients other than thiamine and riboflavin in greater amounts than are present in either the nonenriched flour or the enriched flour.

#### SUMMARY

A study was made of the relative thiamine and riboflavin content of whole wheat, nonenriched and enriched flour of common origin and of breads made therefrom. Four hundred and fifty young rats constituting 38 experimental groups were used in the study. All animals were subjected to a 2-week adaptation period before being placed on experiment. The feeding period was of 8 weeks duration unless death of experimental subject intervened. The results, as a whole, show that flour, enriched in accordance with the present formula, is definitely superior to nonenriched flour with respect to thiamine and riboflavin, and is somewhat superior to whole wheat with respect to these vitamins. While the amount of thiamine contributed by ground whole wheat and by enriched flour, when the flours composed 30% of the diet, is only slightly less than that required for optimal growth in young rats, the amount of riboflavin contributed is definitely insufficient. However, the enriched flour was found to contain more riboflavin than the original wheat. Breads made from ground wheat and from enriched flour show good retention of thiamine and riboflavin as indicated by growth tests and by vitamin assays. Dark bread and milk bread, as prepared and used in these studies, were found to be definitely inferior to breads made from ground whole wheat or from enriched flour

as sources of thiamine, and somewhat less inferior as sources of riboflavin.

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## EDITORIAL REVIEW

# EVALUATION OF AMINO ACID REQUIREMENTS BY OBSERVATIONS ON THE CHICK<sup>1</sup>

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ONE FIGURE

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Investigations of amino acid requirements of chicks conducted by the author and others in the Division of Poultry Husbandry of the University of California over a period of several years, have given rise to a mass of data of a very homogenous nature. Most of the data available have met or approached closely to the following specifications: (a) uniformity in breed and strain of chicks used, (b) pre-test rearing period of 10 to 14 days with an excellent practical diet, (c) experimental period of 10 days, (d) protein or equivalent amino acid levels at 20% or more of the diet, (e) diets substantially complete except for the amino acid under investigation.

The objective of this review was, in part, to establish amino acid intake levels to be recommended by the sub-committee on Poultry Nutrition of the National Research Council. During the course of the analysis of these data there have come to light several relationships of quite fundamental interest.

Two principal variables will be considered throughout this discussion, namely, (a) the percentage of the amino acid in the diet, as determined by additions of the crystalline amino

<sup>1</sup> Presented at the eleventh Annual Meeting, American Institute of Nutrition, Chicago, 1947.

acid or by the content of the amino acid in the protein component, and (b) the percentage daily change in body weight of the chicks, which will be referred to simply as rate of gain. Grau ('47a) has studied such factors as forced feeding, body composition, *ad libitum* feeding and body weight changes of chicks. He has come to the conclusion that change in body weight on an *ad libitum feeding* program is the most satisfactory criterion of dietary adequacy in amino acid studies with chicks. Similarly, Hegsted and Worcester ('47) have found with rats that proteins are classified relative to each other with high accuracy by gain in weight alone.

Where it has been shown that D— or DL— forms of the amino acid are efficiently utilized, these forms have been considered equivalent to the L— form. All protein content data are based on percentage N  $\times$  6.25. The manner of evaluating data is illustrated for certain amino acids in tables. Curves relating amino acid content in the diet to rate of gain are presented in figure 1. In the construction of these curves individual points have been given due weight on the basis of numbers of chicks used in the experiment. Data have not been used for one amino acid when it is apparent that another amino acid deficiency is also exerting an effect in the same region of growth rate. Because of limitations of space, not all of the figures and tables originally prepared can be presented here. A sufficient number of each are given to illustrate the findings in this study.

### *Arginine*

The data for arginine given in table 1 and plotted in figure 1 fall substantially on a straight line over the range of 0.17% to 1.2% arginine, in diets containing whole proteins. It formerly appeared that this requirement was approximately 1% of the diet (Klose et al., '38). However, the upper arginine curve of figure 1 in the region of maximal rate of gain, which is at least 7%, indicates that the optimal arginine content of the diet may be nearer to 1.2%. The question has been raised of the degree of availability of arginine in casein (see Klose

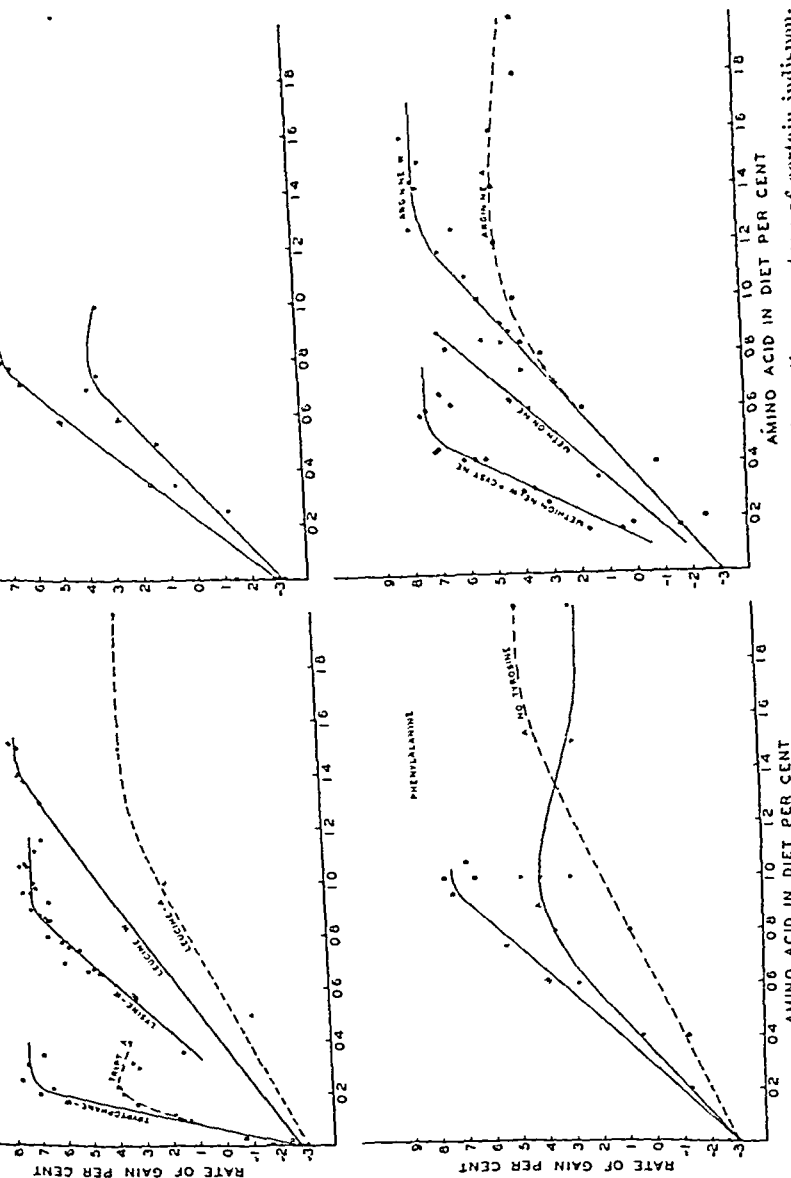


Fig. 1 The relation between daily rate of gain in body weight of chicks to the percentage of certain indispensable amino acids in the diet. Curves marked "W" refer to whole proteins diets . . . those marked "A" to diets containing amino acids or hydrolysed proteins in place of whole proteins.







et al., '38), and if it should be proven that this availability is not complete, the optimal arginine requirement will be somewhat lower than indicated in this report. The lower arginine curve based upon data from amino acids or diets with hydrolysed-protein reaches a maximal gain at approximately the same percentage of arginine in the diet. Both arginine curves extend to the vicinity of a —3% rate at complete deficiency; this is consistent with similar curves for the other indispensable amino acids.

### *Methionine*

It is well-known that the methionine requirement cannot be considered without giving attention to the cystine intake, since the latter is not only synthesized at the expense of the former, but also has a valuable "sparing action" on methionine.

The available data for methionine have been classified in 2 ways, namely, (a) with more than the maximal cystine requirement present, and (b) with substantially no cystine present (table 2). The corresponding curves are also given in figure 1. The curves are practically straight lines up to the region of optimal gain. Both methionine curves extrapolate into the zone of negative gains to an intercept close to —3%.

In the presence of an adequate cystine level in the diet the methionine requirement for optimal gain is close to 0.5%; while the requirement in the relative absence of cystine is close to 0.9% of the diet. These estimates remain in agreement with previously suggested requirements (Almquist, '45).

### *Lysine*

The data on lysine are remarkably consistent. The curve (fig. 1) attains an upper limit for growth at close to 0.9% lysine in the diet (Almquist and Mecchi, '42). All of the data are from diets based upon whole proteins, but these are of a considerable variety, as will be seen from table 3. The linear portion of the curve (fig. 1) extrapolated to zero lysine would reach the vicinity of —3%.



TABLE 2 (continued)

AMINO ACID SOURCES IN DIET	PROTEIN SUPPLIED TO DIET	METHIONINE IN PROTEIN	L-OR DL- METHIONINE TO DIET	TOTAL L-OR DL- METHIONINE IN DIET	RATES OF GAIN	AVERAGE RATE	REFERENCE
	%	%	%	%	%	%	
Corn gluten meal	20	2.1	0.42	0.42	5.9, 6.2	6.1	Gran ('46)
Arginine, lysine							
Cystine, tryptophane							
Glycine, valine							
Cottonseed meal	20	2.1	0.42	0.42	5.7, 5.7	5.7	
Lysine							
Above plus methionine			0.20	0.62	6.9, 6.3, 6.2	6.5	
			0.40	0.82	6.6	6.6	
Peanut meal, lysine	20	1.3	0.26	0.26	3.1	3.1	
Above plus methionine			0.20	0.46	7.0	7.0	
			0.40	0.66	6.9, 6.4, 7.5	6.9	
Fish meal	20	3.0	0.60	0.60	7.4	7.4	Almquist ('46)
Substantially no cystine, <sup>1</sup> adequate choline present.							
Isolated soybean	20	1.5	0.30	0.35 <sup>1</sup>	1.1	1.1	Gran and Almquist ('43)
protein							
Above plus methionine			0.25	0.60	3.7, 3.7, 3.8	3.7	
			0.50	0.85	5.3	5.3	
Casein	20	3.6	0.72				Almquist and Meechi
Gelatin	10	0.6	0.06	0.89 <sup>1</sup>	6.7, 7.0, 6.8, 7.0	6.8	( '41)

<sup>1</sup> Traces of cystine in the diets have been added to the methionine.

*Relation of lysine in the diet to the rate of gain of chicks.*

AMINO ACID SOURCES IN DIET	PROTEIN SUPPLIED TO DIET	LYSINE IN PROTEIN	LYSINE SUPPLIED TO DIET	TOTAL LYSINE IN DIET	PERCENT OF GAIN	AVERAGE RATE	REFERENCE
	%	%	%	%	%	%	
Zinc	30	0.2	0.06	0.06	—1.7	—1.7	Almquist and Meechi ( '12)
Arginine, methionine, threonine, tryptophane, glycine			0.40	0.40	0.2	0.2	
Above plus lysine			0.80	0.80	6.4	6.4	
Edestin	25	2.3	0.58	0.58	3.3	3.3	
Glycine, methionine			0.10	0.08	4.7	4.7	
Above plus lysine			0.20	0.78	5.9	5.9	
			0.30	0.88	6.7	6.7	
			0.40	0.98	6.8	6.8	
Edestin	25	2.3	0.58		6.9	6.9	
Cascia	5	8.5	0.42	1.00			Gran et al. ( '46)
Sesame meal	20	2.8	0.56	0.56	3.2	3.2	
Above plus lysine			0.10	0.66	4.4	4.4	
			0.20	0.76	5.6	5.6	
			0.30	0.86	6.3	6.3	
			0.10	0.96	7.3	7.3	
Arachin	23	1.5	0.34				Almquist et al. ( '43)
Gelatin	7	4.4	0.31				
Lysine			0.10	0.75	5.2	5.2	
Methionine, cystine, tryptophane, valine							

TABLE 3 (continued)

AMINO ACID SOURCES IN DIET	PROTEIN SUPPLIED TO DIET %	LYSINE IN PROTEIN %	LYSINE SUPPLIED TO DIET %	TOTAL L-LYSINE IN DIET %	RATES OF GAIN %	AVERAGE RATE %	REFERENCE
Arachin	18	1.5	0.27				Klose and Almquist ( '41)
Gelatin	5	4.4	0.22				
Yeast	3	6.8	0.20				
Alfalfa	0.2	5.0	0.01	0.70	5.8	5.8	
Tryptophane, threonine, methionine							
Sesame meal	20	2.8	0.56	0.56	3.2	3.2	Grau and Almquist ( '44c)
Above plus lysine			0.50	1.06	7.4	7.4	
Soybean meal	10	5.8	0.58				Almquist and Grau ( '44a)
Sesame meal	10	2.8	0.28	0.86	6.4	6.4	
Soybean meal	13.3	5.8	0.77				
Sesame meal	6.7	2.8	0.19	0.96	7.0	7.0	
Corn gluten meal	20	1.8	0.36	0.36	1.5	1.5	Grau ( '46)
Arginine, cystine, tryptophane, glycine							
Cottonseed meal	20	3.3	0.66	0.66	4.4, 4.3	4.4	
Above plus lysine			0.37	0.93	6.2, 6.3	6.3	
			0.46	1.12	6.6, 6.9	6.8	
Peanut meal	20	3.0	0.60	0.60	5.5	5.5	
Methionine, threonine Tryptophane							
Above plus lysine			0.46	1.06	6.9, 7.0, 7.5	7.1	
Water-extracted linseed meal	20.3	3.3					

### *Tryptophane*

The tryptophane data (table 4) are based upon diets with whole proteins, hydrolysed proteins and amino acids. The curve for whole proteins (fig. 1) extends from an optimal gain at 0.25% *l*-tryptophane (Almquist, '45) down toward the same limiting value common to the other amino acids. The data from amino acid or hydrolysed-protein diets form a separate curve at the higher region of gain, which, however, agrees with the other curve as to the point at which further additions of tryptophane do not cause further increases in growth rate.

### *Leucine*

The leucine curve at 0% leucine is definitely located by experimentally determined rates of — 2.7 to — 3.0% (fig. 1) (Almquist and Grau, '44b). At the upper end the curve is located only by the minimum contents of leucine known to be present in several whole protein sources which, with necessary supplements, have been shown to support good gains. Hence, the leucine requirement for optimal gain does not exceed 1.4% of the diet. The data from amino acid diets (Grau and Peterson, '46), although lower in magnitude of growth, pass through the same maximum region at 1.4% leucine.

### *Phenylalanine.*

Like methionine, phenylalanine may be spared by another amino acid, in this case tyrosine. The data (Almquist and Grau, '44b; Grau, '47a) have been separated on the basis of adequate tyrosine or no tyrosine present in the basal diets. This procedure establishes 2 curves. The first, for adequate tyrosine present, goes through a maximum at close to 0.9%; while the second curve, for no tyrosine present, attains a maximum at approximately 1.6% of the diet. The ends of both of these curves at 0% phenylalanine reach a determined growth rate near — 3%.

A probable curve for whole protein diets has been indicated also. The lower end of this curve should obviously be near

TABLE 4

*Relation of tryptophane in the diet to the rate of gain of chicks.*

AMINO ACID SOURCES IN DIET	PROTEIN SUPPLIED TO DIET	TRYPTO- PHANE IN PROTEIN	L-TRYPTO- PHANE SUPPLIED TO DIET	TOTAL L-TRYPTO- PHANE IN DIET	RATES OF GAIN	AVERAGE RATE	REFERENCE
%	%	%	%	%	%	%	
Hydrolysed casein	20	0.0	0.0	0.01	-2.4,-2.2	-2.3	Almquist and Meeli ('41)
Gelatin	10	0.1	0.01	0.16	3.3	3.3	
Above plus tryptophane			0.15				
Hydrolysed casein	18	0.0	0.0				Klose et al. ('38)
Polished rice	2.4	1.3	0.03		-0.7	-0.7	
Cystine							
Above plus tryptophane							
Casein	18	1.2	0.20	0.23	3.9,4.1	4.0	
Polished rice	2.4	1.3	0.03	0.25	7.3,8.1	7.7	
Cystine, arginine							
Casein	27	1.2	0.32				
Polished rice	2.4	1.3	0.03	0.35	6.3,6.5,7.0,7.2	6.8	
Cystine							
Corn gluten meal	20	0.5	0.10	0.10	1.7	1.7	Grau ('46)
Arginine, lysine, cystine, glycine							
Peanut meal	20	1.0	0.20	0.20	7.0	7.0	
Lysine, methionine, threonine							
Cottonseed meal	20	1.1	0.22	0.22	6.9,6.3,6.2	6.5	
Lysine, methionine							
Hydrolysed casein	15	0.0	0.0				Grau and Ahm- quist ('43)
Gelatin	10	0.1	0.01	0.01	-2.3	-2.3	
Above plus tryptophane			0.25	0.26	3.8	3.8	
			0.50	0.51	3.5	3.5	
Amino acid mixture	20	0.0	0.10	0.10	1.3,1.2,1.2	1.2	Grau and Almquist ( '44b)
Above plus L- or DL- tryptophane			0.20	0.20	3.8,3.8,3.3	3.6	
Zein			0.30	0.30	2.9,3.5	3.2	
Lysine, methionine			0.40	0.40	3.8,3.2	3.5	
Valine, threonine	20	0.1	0.02	0.02	-2.7	-2.7	
Cystine, gelatin							

Fish meal



—3%. The upper end is fixed, by the virtue of growth rates attained with whole proteins of comparatively low phenylalanine content, at not more than 0.9%.

### *Threonine*

Data on threonine requirement are few (Almquist and Grau, '44b; Grau, '47b). At 0% threonine the curve is definitely anchored in the region of —2 to —3%. The points are, for the most part, based upon very small groups of chicks. However, the general characteristics of the curves resemble those obtained with other indispensable amino acids. A similar linear relation has, therefore, been assumed. The optimal threonine content in amino acids diets is apparently close to 0.6%. Certain proteins of comparatively low reported threonine content were found capable of supporting optimal chick growth when providing a dietary threonine level of no more than 0.6%. Hence it seems probable that the estimated requirement is correct in both kinds of diets.

### *Valine*

The amino acids diets (Grau and Peterson, '46) attain a maximal growth-promoting value at approximately 0.8% valine. Whole proteins of comparatively low valine content will support optimal gains when furnishing not more than 0.8% valine to the diet. Hence the 0.8% value may be provisionally accepted as the requirement. The lower ends of the curves are experimentally located close to —3% (Almquist and Grau, '44b; Grau and Peterson, '46).

### *Isoleucine*

The above story is repeated in the case of isoleucine, with the exception that the curve for amino acids diets seems to be displaced slightly toward the left. It is probable, however, that the natural leucine used in the basal diets for this work (Grau and Peterson, '46) may have contained small amounts of isoleucine; as little as 5% would have caused the displace-

ment noted. It is suggested, provisionally, that the isoleucine requirement is close to 0.6% of the diet.

### *Histidine*

The data for histidine are so scarce that no curves could be constructed. On a histidine-free amino acid diet a few chicks lost weight at approximately —2%. A supplement of 0.15% L-histidine may not have been entirely adequate for maximal growth on this diet (Almquist and Grau, '44b). The upper limit of histidine requirement is fixed at not more than 0.30% of the diet. This figure is based on the reported analyses of sources of proteins which have been shown to support optimal growth.

### *The significance of negative gains*

Although protein synthesis in the organism is completely prevented by the lack of an indispensable unit, the processes of protein degradation are not simultaneously stopped. Whether the animal will exhibit loss or gain of protein will depend upon the balance between protein synthesis and protein destruction, both of which are proceeding rapidly, according to modern theory. It is evident from the figures that the complete absence of a truly indispensable amino acid from the diet of the chick results in a loss of weight at a rate in the zone of —2 to —3%. With sufficiently accurate data on all the amino acids in question, it is probable that this zone would have been narrowed toward some value closer to —3. In a few cases where the limiting value is not available from direct experiment, it has been assumed to equal —3% for the purposes of constructing the curves. This assumption is adequately justified from the nature of the data.

The magnitude of the loss of weight is, of course, determined by many factors, such as age and condition of the animal, duration of the deficiency and general experimental procedure, all of which have been comparable in the body of data reviewed. There is nothing fundamental about the pre-

cise numerical value of the limiting negative rate, but its evident constancy under the experimental conditions can only be regarded as of real fundamental significance.

It is obvious that a complete deficiency of any one indispensable amino acid will prevent any protein synthesis in the animal just as absolutely as a complete deficiency of protein in the diet. As a matter of fact, a protein-free diet causes approximately the same rate of weight loss (Grau, '47a). Hence, all these various deficiencies must approach a common limit in effect as the degree of interference with protein synthesis approaches completeness.

In experiments on an entirely different problem (Berg and Rohse, '47) young rats fed gelatin, casein, hydrolysed casein and protein-free diets showed equivalent rates of loss in weight when subjected to a complete deficiency of an indispensable amino acid, or of protein. Rate of growth of rats on a tryptophane-supplemented acid-hydrolysed casein diet was distinctly lower than that on a whole casein diet. These results with rats agree with the general features of the chick results reviewed in the present paper.

It is evident that the point from which to measure growth (as affected by protein synthesis) is not the usually accepted point of zero growth, but some value akin to the — 3% limit of the present data. Any rate significantly more positive than the negative limiting value, even though still negative in sign, is evidence that some protein synthesis is taking place. If such rate is obtained with a complete deficiency of a certain amino acid, then that amino acid is also being synthesized.

*Amino acid requirements as determined by whole  
proteins and by amino acid mixture diets*

It is noteworthy that in substantially every example in which the maximal growth effect from a particular amino acid has been ascertained with whole-protein diets, as compared to amino acid or hydrolysed-protein diets, the requirement values appear to be in close agreement.

Notwithstanding the fact that the best gains obtained with either hydrolysed proteins or amino acid mixtures are less than the optimal gains to be obtained with whole proteins, it is evident that, in either case, the same relative proportions of amino acids to each other and to the whole diet must be established to secure the maximal gain possible on the particular diet. It is further indicated that quantitative amino acid requirement values should be equally valid whether established with amino acid mixtures or with whole proteins.

It appears from the linear nature of the curves and the agreement of the whole proteins and amino acids diets maxima that the requirement of any indispensable amino acid for any rate of growth has a fixed proportion to the others in the diet. This is compatible with the general concept that protein synthesized by an animal is always the same characteristic set of proteins, hence requires the same proportions of materials to be used in the synthesis. The above findings extend this concept to include any rate of synthesis.

These observations further imply that the proportions of the amino acids reaching the synthetic regions in the animal are determined by the proportions in the diet, if not identical with them, since these proportions are the same whether the amino acids come from a variety of whole proteins or are present in the uncombined state, as in amino acids or hydrolysed proteins.

*Growth rate differences observed with complete  
amino acid mixtures and supplemented  
hydrolysed proteins as compared  
to whole proteins*

These differences are a reflection of several possible causes, operating singly or in combination.

*a. Decreased appetite and feed consumption.* Possibly, the more rapid absorption of free amino acids induces a quicker physiological reaction to curtail appetite. This is only a small effect when the amino acid supply is complete, since

then the feed intake is controlled largely by caloric requirements. The marked and almost immediate reduction of feed consumption during a severe amino acid deficiency is perhaps only an extreme stage of the same effect. In such case, the animal cannot as readily dispose of absorbed amino acids by combining them into protein, and is even more quickly satiated. But when the lacking indispensable amino acid is restored to the diet, even though such addition could hardly affect the sensory acceptability of the diet, the increase in the appetite of the animal is very evident.

TABLE 5

*The effect of periodical access to feed on the growth of chicks fed diets containing whole protein as compared to the hydrolysed protein. (20% protein in all diets. No other sources of protein in diets.)*

SOURCE OF PROTEIN	ACCESS TO FEED	FEED EATEN PER DAY PER CHICK	AVERAGE WEIGHT GAIN PER DAY	GAIN-FEED RATIO
		gm	%	
Fish protein	<i>ad libitum</i>	22.9	4.0	.40
Fish protein }	2 hrs. A.M.	21.4	4.0	.42
	2 hrs. P.M.			
Hydrolysed fish protein	<i>ad libitum</i>	20.9	3.5	.34
Hydrolysed fish protein }	2 hrs. A.M.	16.0	2.4	.26
	2 hrs. P.M.			

In an attempt to demonstrate the effect of appetite curtailment by amino acid mixtures in the diet, an experiment was conducted with chicks fed a diet containing fish protein or a hydrolysate of this protein as the exclusive source of amino acids in the diets. Preliminary observations indicated that an appetite-curtailling effect of hydrolysed protein might be to some extent counteracted by a tendency of the chicken to eat more frequently, if less at any one time. Consequently, groups allowed only periodical access to feed were added to the program. The results are given in table 5.

These results show that the chicks having periodical access to the whole-protein diet were able to consume almost as much

feed and to grow as well as the *ad libitum* fed group. The *ad libitum* group on the hydrolysed-protein diet was able to consume only slightly less feed than the above-mentioned groups, but grew less efficiently on the diet — this is the usual result in such comparisons (Klose et al., '38; Stokstad, '40) and is probably a demonstration of causes listed below. However, the periodical-access group fed hydrolysed protein would not ingest sufficient feed to enable this group to keep pace with the others. This difference is most probably a demonstration of a rapidly effective appetite-curtailling action of the hydrolysed protein. Very similar results were obtained in the same experiment performed with young rats.

*b. Increased metabolic destruction of the more rapidly absorbed free amino acids.* These may enter the blood stream faster than they can be diverted into protein synthesis, and the surplus undergoes a relatively greater degree of catabolism than would be the case with amino acids whose release depends upon slow enzymatic digestion of protein.

*c. Increased loss of the rapidly absorbed free amino acids in the urine.*

*d. Increased destruction of amino acids by intestinal bacteria.*

*e. The release during the digestion of whole proteins of some simple peptides which may be used directly by the animal in the synthesis of its own characteristic proteins, thus, perhaps, promoting faster protein synthesis than would be the case following ingestion of free amino acids.*

*f. The presence of a "growth factor" in whole animal proteins, and its absence from amino acid mixtures or completely hydrolysed proteins (Wooley, '45).* In regard to the application of such a "factor" to the results of the present study it can only be said that the animals showed continuous, linear growth curves over the time intervals employed, and at no time any indications of increasing depletion in any growth factor. Furthermore, it was found that the decrease in growth-promoting value of a protein upon hydrolysis (corrected for any amino acid losses) was related most directly to the degree

of hydrolysis, and there was no extra value to be found from leaving a few per cent of whole protein in the diet (animal protein factor). There are numerous examples in which the animals showed optimal gains without animal proteins in the diet.

*Amino acid requirements to support any rate of growth below the optimal*

These may presumably be taken by a horizontal projection across the curves. Thus, for example, the theoretical "maintenance" requirements of the young chick fed a diet containing whole proteins only would be obtained as the intersection of the zero gain level with each of the appropriate curves. In each case the "maintenance" requirement is approximately 30% of the optimal requirement, for the chicks employed in the studies reviewed. This has been experimentally confirmed by feeding diets so low in protein that only the maintenance requirement of several indispensable amino acids was provided.

The curves for the amino acids requirements could be expressed empirically as follows:

$$R = K_1 P - K_2$$

Where  $R$  = rate of change in body weight

$K_1$  = rate constant for change in body weight due to protein synthesis = approximately 10

$P$  = fraction of the optimal level of the amino acid in the diet.

$K_2$  = rate constant for change in body weight due to protein degradation = approximately -3.

It is evident that meeting only half the requirement for a particular indispensable amino acid will not permit half the optimal rate of growth, but only 2/7 of this optimal rate.

#### SUMMARY

1. The level of an indispensable amino acid in the diet bears a linear relation to the rate of growth of the chick, from rapid loss of weight at complete deficiency to maximal growth at complete adequacy of the amino acid.

2. All the curves converge toward a common negative rate of gain at complete deficiencies of any indispensable amino acids, since protein synthesis must stop in all such cases.

3. Net gain of the chick depends upon the balance between protein synthesis and protein catabolism, over the entire range of the curves.

4. While chicks grow better on diets in which the amino acids are supplied chiefly in whole proteins, as compared to diets compounded with amino acids or hydrolysed proteins, the maximal requirements determined with these 2 kinds of diets are in good agreement. This is compatible with the concept that on either kind of diet the chick must synthesize the same body proteins, hence requires the same proportions of amino acids for this purpose.

5. These proportions of indispensable amino acids to each other remain the same for any sub-optimal rate of growth, even in the negative zone of growth.

6. Reasons for the differences in the growth supported by whole proteins as compared to hydrolysed proteins and amino acids are discussed.

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# PRESERVATION OF CAROTENE IN DEHYDRATED VEGETABLES<sup>1</sup>

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Recently we showed (Halverson and Hart, '47) that carotene could be completely preserved in dehydrated alfalfa and cereal grasses if the material had a water content of 12–15% and was sealed in air-tight receptacles. The phenomenon of preservation involves primarily the utilization of the oxygen with production of carbon dioxide — an atmosphere in which carotene is stable. Respiratory enzymes in the dehydrated material are accelerated in action by raising the water content above that usually contained in dehydrated vegetable materials. If the water content of the material is less than 12%, say 10% or less, then the rate of oxygen utilization and carbon dioxide production is reduced with a variable and not always predictable carotene loss.

To test the application of this method of carotene preservation to dehydrated vegetables we used carrots, spinach (leaf) and broccoli. These materials were purchased fresh, dried at 50°C. and then ground to a fine powder. After drying and grinding the water content was as follows: carrots 10.25%, broccoli 8.00%, and spinach 4.5%. The materials were then placed in pint size cardboard boxes with or without water

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adjustment to higher levels and with or without flexo-wax sealing. The sealing was done to prevent loss of carbon dioxide and access of oxygen — a condition absolutely necessary for carotene preservation, and one which we have found effective in the case of dehydrated alfalfa or cereal grasses. Any receptacle that is air-tight such as glass, metal, cellophane, fabricated paper or cardboard dipped in a non-cracking wax should prove effective.

After preparing the materials they were stored at room temperatures of 22–25°C. for 3 months. At the end of that time carotene determinations were made. The chromatographic method of Wilkes ('46) was used. The results are given in table 1.

The original carotene content of the dehydrated materials on a water-free basis in  $\mu\text{g}$  per gm was as follows: carrots 510, broccoli 74.4 and spinach 266.

As expected from our experiments with dehydrated alfalfa and cereal grasses, the carotene loss in 3 months was high in the carrots and broccoli without sealing but not so high in the case of spinach under the same conditions of storage and with the low water content of 4.5%. Under sealed conditions the carotene loss in all the materials decreased in general with increase in water content. With 12.5% of water the losses in all the materials were negligible.

It is apparent from the data that in these dehydrated products the carotene loss can be greatly reduced with sealed conditions and a total water content of 10–12.5%.

In table 1 are also brought together observations on the color, taste, and internal gas pressure as it may have developed. With dehydrated alfalfa and cereal grasses sealed, and with the water content up to 20% no internal gas pressure, involving bulging of the carton storage receptacles, has been observed. This same situation was observed with spinach, but in the case of carrots and broccoli some internal gas pressure developed particularly at the higher water levels. Also darkening of the product, especially in the case of carrots, was observed. To reduce the possibility of gas pressure, as in the

TABLE 1  
*Observations on character of dehydrated products and  
 carotene losses in 3 months' storage at 22-25°C.*

TREATMENT	COLOR	TASTE	AROMA	GAS PRESSURE	CAROTENE CONTENT, $\mu\text{g}/\text{gm}$ WATER FREE BASIS			
					% water	Initial	3 months	% loss
Carrots — dehydrated								
No seal	Reddish brown	Sweet	Pleasant	Negative	10.2	510	150	71.0
Sealed	Reddish brown	Sweet	Pleasant	Negative	10.2	510	361	29.0
Sealed	Deep brown	Bland	Pleasant	Positive	12.5	510	442	13.0
Sealed	Deep brown	Bland	Coffee-like	Positive	15.0	510	469	8.0
Broccoli — dehydrated								
No seal	Green color	Bland	Pleasant	Negative	8.0	74.4	36.5	51.0
Sealed	Green color	Bland	Strong broccoli	Negative	8.0	74.4	70.0	6.0
Sealed	Olive green	Bland	Strong broccoli	Positive	12.5	74.4	73.2	1.6
Sealed	Olive green	Bland	Strong broccoli	Positive	15.0	74.4	72.6	2.0
Spinach — dehydrated								
No seal	Bright green	Bland	Pleasant	Negative	4.5	266	215	19.0
Sealed	Bright green	Bland	Pleasant	Negative	4.5	266	236	11.0
Sealed	Bright green	Bland	Pleasant	Negative	10.0	266	260	2.0
Sealed	Olive green	Bland	Pleasant	Negative	12.5	266	261	1.8
Sealed	Olive green	Bland	Pleasant	Negative	15.0	266	252	5.2

case of carrots and broccoli with water levels of 12.5%, the water level could be held at 8-10%. Under such conditions complete carotene preservation may not be obtained, but the loss of carotene under sealed conditions would be greatly reduced. Apparently the application of the principle of a proper water content with sealed storage can be made use of for carotene preservation in dehydrated fresh vegetable tissues. In the case of spinach a water content of 10% plus sealing reduces the carotene loss to practically zero. Whether this observation would be general for spinach is unknown. Usually with other dehydrated plant tissues such as alfalfa, rye, oats and carrots a moisture content of 10% or less does not give a maximum carotene retention observed with moisture levels of 12-15%.

The color of the stored products deepened somewhat with increased water content. However, at 10-12% of total water the color was not greatly different from that of the original product. With 15% of total water the color of the carrots became a deep brown and in the other 2 products an olive green. Broadly speaking, a water content of 10-12% and sealing will not only preserve well the carotene but also give a product resembling the original unsealed material in color, taste and aroma. It is probable that the moisture levels required for maximum carotene preservation will vary with the different vegetables and the method of dehydration employed.

#### SUMMARY

1. The carotene content of dehydrated spinach, broccoli and carrots was well preserved under storage at room temperature for 3 months by sealing in cardboard receptacles and adjusting the total water content to 10-12%. In the case of carrots and broccoli with the total water content of 12.5% some internal gas pressure developed. With 8-10% of total water no internal gas pressure developed but the carotene loss was greater than at the higher water level.

2. Under sealed conditions of storage with a total water content of 8-10% the taste, aroma and color of the original product were well retained.

3. The principle of preservation involved is mainly the acceleration of the respiratory activity of enzymes present in the dried plant tissue with the result that the oxygen present is replaced with carbon dioxide.

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# INTERRELATION BETWEEN $\alpha$ -TOCOPHEROL AND PROTEIN METABOLISM

## II. THE INCREASED UTILIZATION OF CASEIN PRODUCED BY $\alpha$ -TOCOPHEROL, YEAST DIGEST, OR XANTHINE IN THE RAT-GROWTH PROTEIN-QUALITY TEST<sup>1</sup>

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ONE FIGURE

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In a previous paper (Hove, '46) it was shown that d,  $\alpha$ -tocopherol reduced weight loss in adult rats maintained on a 5% crude-casein diet for extended periods of time. It was pointed out that this result was in apparent contradiction to the work of Dam ('44) in which was noted no influence of  $\alpha$ -tocopherol on the body weight of rats maintained until death on a diet containing approximately 5% protein from yeast. It occurred to us that the 4-week, rat-growth method originated by Osborne, Mendel and Ferry ('19) for determining protein quality might be useful in resolving these divergent results.

### EXPERIMENTAL

Litter-mate pairs of male albino rats were placed on the test diets at weaning. One member of each pair received daily, 1 mg of d,  $\alpha$ -tocopherol in 2 drops (50 mg) of olive oil, while the other received only the olive oil. Body weight and food consumption records were made weekly for the second to fifth

<sup>1</sup> Communication no. 119 from the Laboratories of Distillation Products, Inc., Rochester, N. Y.

week from weaning. The protein-efficiency ratios were obtained by dividing the 4-week body-weight gain by the total 4-week protein intake. The composition of the weight gains was not determined. Therefore the effect of tocopherol to be described may have resulted from increased deposition of fat or other constituents, rather than increased protein utilization. However, the custom that has grown up around the use of this method will be followed and results will be expressed as protein efficiency rather than more correctly as food efficiency.

TABLE 1  
*Composition of diets used.*

BASAL MIXTURE	PROTEIN SOURCE SALT MIXTURE, USP XII, NO. 2 <sup>1</sup> LARD	SUCROSE % 56 1 10
Vitamin supplements		<i>per gm of diet</i>
	Thiamine-HCl	10 µg
	Riboflavin	10 µg
	Pyridoxine HCl	10 µg
	Calcium pantothenate	25 µg
	Nicotinic acid	50 µg
	2-Methyl, 1-4 naphthoquinone	5 µg
	Choline chloride	1.0 mg
	<i>D</i> -Inositol	0.1 mg
	Vitamin A	50 units
	Vitamin D (Delsterol)	3 units

<sup>1</sup> With added ZnCO<sub>3</sub>, 0.1%; CuSO<sub>4</sub>·7 H<sub>2</sub>O, 0.08%; and MnSO<sub>4</sub>, 0.01%.

*Ad libitum* feeding was allowed in most instances. Diets were weighed into a "Franke" earthenware cup. This cup was contained in a straight-sided aluminum dish. Using this system food spillage was reduced to less than 3 gm weekly for nearly all rats. The spillage was determined by placing collecting papers under the cages and weighing the discard weekly.

The composition of the diets is indicated in table 1. The B-vitamins<sup>2</sup> and vitamin K were thoroughly ground with a small quantity of purified casein or sucrose, as the case might

<sup>2</sup> Merck.

be. Vitamins A and D were added to the lard. Additions of folic acid and biotin have not been made to the diets. The diets were kept refrigerated at all times.

The casein used was a "vitamin-test" product<sup>3</sup> and was assumed for our purpose to be 100% protein. A yeast-protein concentrate<sup>4</sup> prepared by enzymatic digestion was used; this contained 67% protein. The washed wheat gluten contained 70% protein.

In the first experiment casein was used at 5 and 10% levels both with or without the addition of 0.5% *l*-cystine to the diet. The yeast protein was used to the extent of 10 and 15% in the diet, and wheat gluten protein at 15% in the diet. The results are shown in table 2. On the 10% casein diets the  $\alpha$ -tocopherol-supplemented rats showed protein efficiency ratios significantly greater than those of the controls. On none of the other diets was there a benefit due to tocopherol.

Some of the groups from this experiment were continued on their diets for 12 weeks at which time the tooth pigment of the maxillary incisors was rated on a 0 to 4 scale. The rats were autopsied and examined for stomach ulcers. The results given in table 3 are similar to those reported previously for casein (Hove, '46). Yeast protein apparently can replace tocopherol as a protective agent against depigmentation.

Furthermore, it will be noted from table 3 that tocopherol supplementation failed to induce an enhanced weight gain when yeast supplied the protein of the diet. Dam's report ('44) in which tocopherol failed to influence body weight and the opposite finding by Hove ('46) that tocopherol had a very significant effect on body weight may now be reconciled. Dam used yeast protein and Hove used casein.

A second experiment was designed to repeat the tocopherol effect on the 10% casein diet and to determine the influence of small amounts of yeast protein on the utilization of casein.

<sup>3</sup> General Biochemicals, Inc.

<sup>4</sup> "Basamin-Busch" from Anheuser-Busch, Inc., St. Louis, Mo.

TABLE 2

*Effect of d,  $\alpha$ -tocopherol on the utilization of protein by growing rats. Six litter-pairs used per diet. Values are the 4-week averages from the second through the fifth week after weaning.*

PROTEIN IN DIET	d-TOCOPHEROL FED DAILY	AVERAGE GROWTH	AVERAGE FOOD CONSUMED	PROTEIN EFFICIENCY RATIO (GROWTH/PROTEIN INTAKE)	$t = \frac{D^2}{E_d}$
	mg	gm	gm	$\pm$ S.E. <sup>1</sup>	
Casein, 5%	0	10.3	156	$1.31 \pm .115$	.034
	1	9.2	145	$1.26 \pm .094$	
Casein, 5% + 0.5% l-cystine	0	15.5	157	$1.76 \pm .148$	0.39
	1	15.5	171	$1.68 \pm .142$	
Casein, 10%	0	39.7	226	$1.73 \pm .095$	2.26
	1	51.3	259	$1.99 \pm .066$	
Casein, 10% + 0.5% l-cystine	0	87.5	301	$2.72 \pm .084$	2.09
	1	96.8	311	$2.96 \pm .079$	
Yeast protein, 10%	0	38.0	240	$1.58 \pm .095$	0.57
	1	38.3	228	$1.67 \pm .124$	
Yeast protein, 15%	0	86.2	320	$1.77 \pm .103$	0.57
	1	88.9	313	$1.86 \pm .120$	
Wheat gluten protein, 15%	0	22.1	170	$0.87 \pm .031$	2.24
	1	19.7	183	$0.73 \pm .054$	

$$^1 \text{S. E.} = \sqrt{\frac{\text{dev.}^2}{N(N-1)}}$$

<sup>2</sup>  $t$  for  $P = .05$  is 2.23. Values greater than this are significant.

TABLE 3

*Growth, stomach ulcers, and color of maxillary incisor teeth of rats after 12 weeks on various protein diets.*

PROTEIN	DAILY d-TOCOPHEROL	AVERAGE WEIGHT GAIN (12 WEEKS)	STOMACH ULCER INCIDENCE	AVERAGE TOOTH COLOR <sup>1</sup>
	mg	gm	%	
Casein, 5%	0	15	66	0.4
	1	18	17	2.6
Casein, 10%	0	117	0	0.5
	1	156	0	2.9
Yeast protein, 10%	0	121	0	1.1
	1	126	0	3.1
Wheat gluten protein, 15%	0	64	33	2.5
	1	60	17	3.3

<sup>1</sup> A score of 0 = a tooth completely bleached; chalk white.

A score of 4 = a tooth normally colored; brownish yellow.

The results of this experiment are shown in tables 4 and 5. With the larger number of animals the influence of  $\alpha$ -tocopherol on utilization of protein on the 10% casein diet was more striking. Statistically the difference due to tocopherol is significant with a probability of more than 99 chances in 100

TABLE 4

*Effect of 1 mg d,  $\alpha$ -tocopherol daily on the utilization of casein at a 10% level in the diet of young male rats. Values are 4-week averages.*

EXPERIMENT NUMBER	NUMBER OF LITTER-MATE PAIRS	WEIGHT GAIN (GM)		PROTEIN INTAKE (GM)		PROTEIN EFFICIENCY RATIO (GAIN/PROTEIN)		
		-E <sup>1</sup>	+E <sup>2</sup>	-E	+E	-E	+E	$t = D/E_4$ <sup>3</sup>
1	6	39.7	51.3	22.6	25.9	1.73	1.99	(3.25)
2	15	50.5	59.2	22.6	23.2	2.23	2.48	(4.27)
3	5	45.2	45.6	24.1	20.3	1.88	2.23	(3.64)
4	5	35.6	46.2	19.3	22.1	1.85	2.09	(3.73)
5	5	38.8	43.2	21.4	20.7	1.81	2.09	(3.74)
Average		42.0	48.9	22.0	22.4	1.88	2.18	

<sup>1</sup> Without tocopherol.

<sup>2</sup> With tocopherol.

<sup>3</sup> Student's method for  $t = D/E_4$  in which values of  $t$  for  $P = .02$  are 3.37, 2.62 and 3.75 for 5, 14 and 4 degrees of freedom, respectively.

TABLE 5

*Effect of yeast-protein digest and xanthine on the utilization of casein for growth by young male rats over a 4-week period.*

EXPERIMENT NUMBER	PROTEIN SOURCE	NUMBER OF RATS	DAILY $\alpha$ -TOCOPHEROL	PROTEIN EFFICIENCY RATIO (GAIN/PROTEIN) $t = D/E_4$ <sup>1</sup> $\pm$ S.E.
2	Casein, 10%	15	mg 0	2.23 $\pm$ .064
	Casein, 8% + 2% yeast protein	10	0	2.56 $\pm$ .063
	Casein, 8% + 2% yeast protein	10	1	2.50 $\pm$ .117
3	Casein, 10%	5	0	1.88 $\pm$ .023
	Casein, 10% + 0.1% xanthine	5	0	2.11 $\pm$ .070
	Casein, 10% + 0.1% xanthine	5	1	2.12 $\pm$ .074

<sup>1</sup> Values for  $t = D/E_4$  by the Fisher method in which values of  $t$  for  $P = .01$  are 2.81 and 3.36 for 23 and 8 degrees of freedom, respectively.

(table 4). However, the addition of a small amount of yeast protein also increased the utilization of casein and completely replaced  $\alpha$ -tocopherol in this action (table 5). The absolute values in this experiment are considerably higher than usual and we have no explanation for this.

Schwarz ('44) has noted that xanthine can replace  $\alpha$ -tocopherol in preventing liver damage and delaying death in rats on an alkali-washed casein diet. Therefore, a third experiment was designed to determine the effect of xanthine on casein utilization. From the results given in table 5 it is evident that xanthine does increase the protein efficiency ratio and replaces  $\alpha$ -tocopherol in producing this effect. Perhaps  $\alpha$ -tocopherol acts at some point in the metabolic changes from dietary amino acids to xanthine. Consequently, in the absence of tocopherol, xanthine becomes a dietary essential.

The effect of d,  $\alpha$ -tocopherol on the utilization of casein at the 10% level in the diet has been confirmed in 2 other brief experiments, the results of which are tabulated in table 4.

In the preceding experiments *ad libitum* feeding has been allowed, using diets containing the single level of 10% protein, for the most part. To furnish a clearer insight into the nature of the tocopherol effect another experiment was set up which used an equalized-feeding technique with diets containing a wide range of casein levels.

Diets with purified casein contents of 6, 8, 10, 12, 14, 18 and 40% were prepared. Five litter-pairs of weanling male albino rats were placed on each diet, and tocopherol supplements given as before. Each rat received exactly 7.0 gm of diet daily. Except for 4 rats on the 6% casein diet all daily allotments were consumed. On the 6% casein diet the total refusal was 48 gm for the —E group and 10 gm for the +E group over the 4 weeks of the experiment. Protein efficiency ratios were calculated in the usual way by dividing the 4-week total gain in body weight by the protein contained in the quantity of diet consumed by each rat during this 4-week period. The results are expressed graphically in figure 1. On the 8 and 10% casein diets the increased utilization due to tocopherol

was statistically significant, the respective  $\frac{D}{E_d}$  values for those groups being 2.96 and 3.55 (Fisher method). No other differences were statistically significant.

EFFECT OF  $\alpha$ -TOCOPHEROL ON CASEIN UTILIZATION  
(EQUALIZED FEEDING TECHNIQUE)

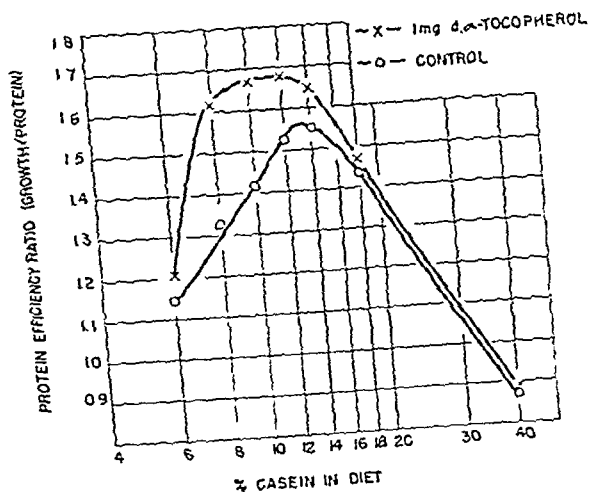


Fig.1 The influence of  $\alpha$ -tocopherol on the protein efficiency ratio as determined on diets with varying casein contents. Each point represents the average of 5 rats over 4 weeks. An equalized feeding technique was used in which all animals received 7.0 gm of diet daily.

#### DISCUSSION

Under the experimental conditions outlined,  $\alpha$ -tocopherol produced a small but significant increase in the efficiency of food utilization by growing rats on a diet containing 10% purified casein. The improvement due to tocopherol was statistically significant in each of 5 consecutive experiments employing a total of 72 rats fed *ad libitum*. The average protein-efficiency ratio for the vitamin E-low groups was 1.88, as compared with 2.18 for the tocopherol supplemented groups. The beneficial effect of  $\alpha$ -tocopherol was as evident on equalized feeding as on *ad libitum* feeding techniques.

This effect of tocopherol in improving efficiency of food utilization has been noticed by Patrick and Morgan ('43) in chickens fed a synthetic diet containing casein protein. An explanation for the beneficial influence of tocopherol on food utilization efficiency or on protein-efficiency ratio may be found in an experiment described by Bosshardt et al. ('46a). As non-protein calorie intake increased, protein intake remaining constant, the protein-efficiency ratio was found to increase. Consequently, in our experiments tocopherol may be increasing the efficiency of fat metabolism thus giving, in effect, a higher calorie intake and resulting indirectly in a higher protein-efficiency ratio.

Low levels of a yeast protein autolysate, or 0.1% of xanthine added to the low-casein diet, each improved the protein-efficiency ratio and completely replaced  $\alpha$ -tocopherol in this action.

The ability of  $\alpha$ -tocopherol or xanthine to improve casein utilization may be related to the report of Schwarz ('44) that these compounds prevented cirrhotic changes in the liver and death of rats on a diet containing 15% alkali-extracted casein. Traces of xanthine contained in crude casein would be removed by the alkali extraction and a xanthine-free diet would result. Giri and Rao ('46) reported that xanthine functions as an antioxidant in metal catalyzed oxidations and it may be in this same capacity that it acts similarly and non-specifically with  $\alpha$ -tocopherol *in vivo*.

Schwarz found dried yeast, 0.5 gm daily, to be but slightly active, while our results showed a yeast protein concentrate to be highly active. The active agent in our yeast preparation may have been liberated or formed from precursors during the enzymatic digestion. Studies on this problem as well as the influence of other purine bodies and of folic acid are being pursued. In this regard Bosshardt et al. ('46b) working with mice noted an increased utilization of casein protein produced by a butanol extract of liver.

On diets with casein levels as low as 5 or 6%, tocopherol did not increase the protein-efficiency ratio over the 4-week



test period. Rats kept on such diets for periods up to 20 weeks, however, showed a dramatic growth and survival benefit from  $\alpha$ -tocopherol (Hove, '46). This difference may indicate that the interrelation between protein and  $\alpha$ -tocopherol follows separate and independent mechanisms under these 2 experimental conditions.

# SUMMARY

1. d,  $\alpha$ -tocopherol increases the utilization of casein protein for growth of young rats when the casein level in the diet is between 6 and 12%. There is no benefit due to tocopherol at casein levels over 12% or under 6% in the 4-week test period.

2. d,  $\alpha$ -tocopherol reduces the incidence of stomach ulcers and the bleaching of maxillary incisors which occur in rats maintained on low-casein diets.

3. A yeast-protein concentrate or 0.1% xanthine in the diet is as effective as  $\alpha$ -tocopherol in increasing the utilization of casein protein.

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# THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

## IV. THE COMPARATIVE COMPOSITION OF RATS IN RELATION TO INTAKE OF FAT AND CALORIES <sup>1</sup>

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Earlier studies from this laboratory have shown beneficial effects on rats of the inclusion of liberal amounts of fat in the diet. Growth, resistance to undernutrition, physical capacity, and reproductive performance were all improved on synthetic diets containing 10 to 40% of fat, as compared with similar diets lacking fat (Deuel et al., '47; Scheer et al., '47a, b, c).

The present report includes data on the content of water, protein, fat, ash, and calcium in the bodies of rats selected from these experiments. The methods used were essentially those employed by Deuel et al. ('44), but the entire body of the animals was analyzed, rather than the eviscerated carcass. The intestine was removed, and the contents were

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The work was also undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions and conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

washed out with saline; the intestine was then passed through the meat grinder together with the remainder of the rat body.

The composition of the diets used and general experimental procedure have been published previously (Deuel et al., '47). Briefly, the diets were composed entirely of purified or synthetic materials, in such proportions that the intake of protein, minerals and vitamins would bear a constant ratio to caloric intake. The protein content of the diet ranged from 25% to 37.5%. The fat contents varied as follows: 60a, no fat, but ethyl laurate (1%) added as carrier for fat-soluble vitamins; 60b, no fat, but methyl linolate (1%); 60c, no fat, but methyl linolate (2.5%); 61, 5% fat (cottonseed oil); 62, 10% fat; 63, 20% fat; 64, 40% fat. The stock diet contained oats, wheat, alfalfa, yeast, salts, and cottonseed oil; the fat content was about 14%, and protein about 15%.

#### RESULTS

The results of the body analyses are summarized in table 1. The values for rats fed *ad libitum* (series I) agree fairly well with the figures of Deuel et al. ('44), but the variability is greater in our values. The protein, ash and calcium contents of our males appear somewhat higher, the carbohydrate somewhat lower than those reported by Deuel et al., and the sex differences reported by these authors are not as evident in our data. In the earlier tests a uniform fat level was fed in all tests and a large number of rats were available for comparison of the influence of sex on body composition. Apparently, however, the inclusion of the viscera in the analysis does not greatly alter the values obtained.

A period of very severe caloric restriction in young adult rats previously fed the stock diet *ad libitum* evidently results in a decrease in fat and carbohydrate, and an increase in water and ash (series II). The protein content increases slightly. The fat content appeared to be slightly higher on diets 63 and 64 providing liberal fat intake than on fat-free or low-fat diets. This is correlated with the fact that mortality was least in animals receiving diets high in fat. How-

TABLE 1

The body composition of male and female rats. Series I were fed ad libitum on diets containing several levels of fat for 18 weeks after weaning; Series II were fed ad libitum on stock diet for 19 weeks, followed by diets containing several levels of fat at 2 cal. per day for 8 weeks and then 12 cal. per day for 4 weeks; Series III received 12 cal. per day for 12 weeks after weaning while Series IV were also fed at a level of 12 cal. per day for 12 weeks after weaning but then received the diets ad libitum for 4-7 weeks (females) or 7-10 weeks (males).

CATEGORY	SERIES	DIET 60a		DIET 60b OR 60c		DIET 61		DIET 62		DIET 63		DIET 64		STOCK DIET	
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Total number analyzed	I II III IV	8	5	8 <sup>1</sup> 3 4 4	5 <sup>1</sup> 5 4 4	7 1 4 3	5 5 4 3	9 3 4 4	5 4 4 1	8 3 3 5	5 4 3 4	8 6 3 5	5 3 4 4	7 6 3 2	5 3 4 5
Body weight, gm	I II III IV	235	179	256 <sup>1</sup> 122 55 183	176 <sup>1</sup> 109 55 153	311 100 62 221	202 111 50 291	326 120 58 230	215 121 55 154	377 145 62 226	223 121 58 168	313 142 66 213	208 108 57 172	310 153 56 191	202 132 51 179
Water, %	I II III IV	58.2	60.6	60.5 <sup>1</sup> 69.4 68.3 65.9	61.5 <sup>1</sup> 69.0 67.3 63.0	57.0 69.9 67.7 66.4	58.8 68.9 61.6 63.1	54.6 68.8 71.9 61.0	50.9 69.8 68.3 63.1	52.2 67.8 68.8 61.0	56.7 66.1 65.1 61.3	55.2 67.4 67.1 68.0	56.8 67.3 66.8 60.7	58.8 69.6 73.0 65.8	59.2 69.1 66.2 61.7
Protein, %	I II III IV	19.6	16.0	19.4 <sup>1</sup> 20.9 17.3 17.1	15.0 <sup>1</sup> 19.9 16.5 15.3	18.7 20.2 16.5 16.6	16.5 19.6 16.9 16.1	17.6 21.6 14.1 17.3	17.4 19.5 16.8 16.1	17.9 21.0 16.2 16.6	16.5 20.5 18.0 16.1	18.7 20.9 20.2 16.3	17.1 20.2 16.2 17.3	18.2 20.5 16.8 17.2	17.4 19.8 16.3 17.1
Fat, %	I II III IV	16.2	18.3	14.2 <sup>1</sup> 1.2 8.1 11.1	16.4 <sup>1</sup> 2.1 9.5 16.5	19.1 1.0 9.1 11.6	19.1 2.1 11.7 11.7	22.5 1.3 8.1 11.7	25.5 3.1 8.4 15.5	21.7 3.2 9.2 13.5	21.7 5.1 9.2 17.1	21.0 3.5 11.9 10.2	20.1 5.3 9.8 16.0	20.1 10.0 4.7 12.0	9.9 1.8 9.0 12.9
Catholydinate, %	I II III IV	2.3	1.6	2.1 <sup>1</sup> 3.5 2.6 2.8	3.8 <sup>1</sup> 3.8 3.1 1.9	1.6 2.3 3.0 2.8	2.0 4.1 3.6 3.8	2.0 2.6 2.0 3.8	3.0 3.3 3.6 1.9	2.0 3.4 2.5 2.8	1.5 2.5 3.9 2.1	2.1 3.0 3.0 2.7	2.2 3.0 3.5 2.1	2.0 2.9 1.8 2.0	10.4 3.3 4.5 1.6
Ash, %	I II III IV	3.7	3.5	3.5 <sup>1</sup> 5.0 3.7 3.1	3.3 <sup>1</sup> 4.9 3.6 3.3	3.3 6.6 3.7 2.6	3.6 5.3 3.2 3.6	3.3 5.7 3.9 2.5	3.2 5.2 3.9 3.0	3.2 4.6 3.9 3.1	3.6 5.0 3.8 3.1	3.0 5.2 3.3 2.8	3.5 4.2 3.7 3.6	3.1 5.2 3.7 3.0	3.3 5.0 4.0 3.7
Calcium, %	I II III IV	1.52	1.60	1.44 <sup>1</sup> 1.94 1.23 0.93	1.66 <sup>1</sup> 1.79 1.15 1.09	1.39 2.20 1.18 0.72	1.70 1.78 1.02 1.02	1.22 1.89 1.16 0.61	1.34 1.45 1.20 1.20	1.38 1.56 0.90 0.93	1.58 1.76 1.33 0.88	1.15 1.77 1.37 0.78	1.50 1.58 1.17 1.05	1.30 1.62 1.15 0.95	1.41 1.52 1.37 1.10

<sup>1</sup> Received diet 60b.

ever, it should be emphasized that mortality was also low on the stock diet, and that the fat content of animals on this diet fell to low levels. In an earlier report (Scheer et al., '47c) we have suggested that the mortality observed was somehow related to vitamin deficiencies.

Rats kept on a restricted caloric intake for 12 weeks after weaning (series III) have a higher content of water, and less fat than do animals fed *ad libitum*; protein content is somewhat lower in the males, but not in females. There is no clear relation of fat content of the body to that of the diet. When these animals on restricted feeding were transferred to *ad libitum* feeding (series IV), there were small decreases in water and ash content, and definite increases in fat content on all diets. The fat content of the body does not appear to be correlated with the level of dietary fat. A slight decrease in carbohydrate is apparent in females, but not in males.

Taken together, our data show a very striking uniformity of body composition with gross variations in fat content of the diet and caloric intake. Fat and water content are the greatest variables, with protein remaining nearly the same throughout. In an earlier report (Deneel et al., '47) the greatest increase in body size in male rats was found on diets containing about 20% fat. This same diet results in the greatest deposition of body fat. However, the differences in weight on the various diets are not simply the result of differences in fat deposition. In fact, the relative contributions of the various components to the increased size, above that on a fat-free diet, are remarkably uniform. Table 2 presents data showing the difference in weight between rats receiving diets with fat and rats fed the fat-free ration (60b), and the percentage contribution to this increase of each of the body components analyzed for, this being determined by dividing the difference in total weight of each component by the difference in animal weight.

An earlier report (Scheer et al., '47b) showed that physical capacity is greater in rats fed liberal amounts of fat than in rats fed the fat-free diet. There is no evidence from the

present results that this difference is related to body composition.

TABLE 2

*Relative contributions of various components of the body to the differences in weight between male rats fed diets containing fat and male rats fed a fat-free diet (60b).*

DIET	WEIGHT DIFFERENCE	CONTRIBUTION TO WEIGHT DIFFERENCE FROM				
		Water	Protein	Fat	Ash	Carbohydrate
	<i>gm</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>
61	55	40	15	43	2	0
62	70	33	10	54	3	0
63	121	35	14	47	2	2
64	87	39	16	42	1	2

## SUMMARY

1. Data are presented on the body composition of rats fed diets varying in fat content from 0 to 40%, at normal and severely restricted levels of caloric intake.

2. In general, the fat content of the body is not related to fat content of the diet; rats receiving diets with 20% fat fed *ad libitum* have the highest content of body fat.

3. Caloric restriction, in weanling or young adult rats, results in relatively high water content and very low fat content. Ash also increases in young adult rats.

4. The greater body size on diets containing liberal amounts of fat as compared with fat-free diets, is not the result solely of deposition of fat.

5. There is no evidence that the effects of varying fat content in the diet on resistance to undernutrition and on physical capacity are the result of differences in gross body composition.

## ACKNOWLEDGMENTS

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# PEAS, SUPPLEMENTED WITH WHEAT GERM OR CORN GERM, AS A SOURCE OF PROTEIN FOR GROWTH<sup>1</sup>

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Legumes are readily obtained sources of vegetable protein and are frequently used in low cost diets. They are often used, also, to complete the protein quota in rations for both man and animals. The available supply of sulfur-containing amino acids limits the value of legumes as the sole source of protein. Woods, Beeson and Bolin ('43) and Lehrer, Woods and Beeson ('47) have shown that the growth efficiency of dry Alaska peas is more than doubled by the single addition of methionine to a 10% protein diet.

The cereal germs are another source of vegetable protein. As early as 1919 Osborne and Mendel reported that the proteins of the embryo of wheat were different chemically from those of the endosperm and were superior to those of the whole grain for maintenance of adult rats as well as for growth in the young. Stare and Hegsted ('44) reviewed the published reports on the protein of both wheat and corn germs and summarized their review by stating that these proteins were of "high nutritive value." Jones and Widness ('45, '46) obtained consistently better growth in weanling rats with wheat germ than with corn germ as the only source of protein.

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Modern milling methods produce both wheat and corn germs which are relatively free from other portions of the grain. These grain germs are available for both human and animal feeding.

The work reported here is a continuation of the study of the use of dry peas as a source of protein. This paper is concerned with the growth of rats produced by the combination of wheat or corn germ with peas.

#### EXPERIMENTAL PROCEDURE

Alaska field peas (23.1% protein) were soaked in distilled water overnight, cooked, dried at 62°C. and then ground. The wheat germ (28.8% protein), the usual commercial product, was ground in the laboratory, but not further processed. The corn germ<sup>3</sup> (23.2% protein) was a "low fat, dehydrated," finely ground product. A diet containing dried whole egg (44.0% protein) as the source of protein was used as a standard of comparison. All diets were made up to contain protein at a 10% level. The complete compositions of the diets are given in table 1.

Twenty-one gm of a vitamin-sucrose mixture and 2.5 gm choline chloride were included in each kg of diet. This amount of vitamin mixture provided the following supplements per 100 gm of diet: thiamine, 0.49 mg; riboflavin, 0.49 mg; pyridoxine, 0.61 mg; nicotinic acid, 0.61 mg; calcium pantothenate, 4.9 mg; para-amino benzoic acid, 29.4 mg; inositol, 98.0 mg; and 250 mg choline chloride. The Osborne and Mendel ('19) salt mixture was used in all diets. Methionine when used replaced an equal amount of sugar.

Weanling rats housed in individual cages were used for the investigation. They were weighed weekly for 8 weeks and food consumption records made at each weighing.<sup>4</sup>

The statistical analyses of the data were made according to the methods of Snedecor ('46).

<sup>3</sup> Generous amounts were furnished by the Vio Bin Corporation, Monticello, Illinois.

<sup>4</sup> The assistance of R. A. York in the care of animals is gratefully acknowledged.

TABLE 1

*Composition of diets.<sup>1</sup> The various sources of protein were used in amounts calculated to furnish protein at a 10% level.*

LOT NO.	PROTEIN		SUCROSE	SALT MIXTURE	COTTON-SEED OIL	COD LIVER OIL
	Source	Amount				
		<i>gm/kg</i>	<i>gm/kg</i>	<i>gm/kg</i>	<i>gm/kg</i>	<i>gm/kg</i>
1	Egg <sup>2</sup>	227	639.5	40	30	20
2	Peas	433	444.5	40	39	20
3	Wheat germ	352	563.5	40	1	20
4	Corn germ	488	400.5	40	28	20
5	Peas	433				
	and		441.5	40	39	20
	dl-methionine	3				
6	50% Peas	195				
	and		512.5	40	14	20
	50% wheat germ	195				
7	75% Peas	310				
	and		481.5	40	22	20
	25% wheat germ	103				
8	87.5% Peas	372				
	and		465.5	40	26	20
	12.5% wheat germ	53				
9	50% Peas	231				
	and		425.5	40	29	20
	50% corn germ	231				
10	75% Peas	337.5				
	and		437.5	40	29	20
	25% corn germ	112.5				
11	87.5% Peas	389				
	and		442	40	30	20
	12.5% corn germ	55.5				

<sup>1</sup> Each diet contained 21 gm/kg of the vitamin-sucrose mixture described in the text in addition to 2.5 gm/kg of choline chloride.

<sup>2</sup> Egg diet contained 20 gm/kg of Agar-Agar.

## RESULTS AND DISCUSSION

The results of these experiments are summarized in tables 2 and 3.

Examination of the results obtained from the diets containing a single product, as sole source of protein, reveals that the cereal germs compared favorably with the whole egg

TABLE 2

*Average growth response.*

LOT NO.	DIET	NO. OF RATS	AV. DAILY GAIN gm	AV. GAIN PER GM OF PROTEIN gm	AV. FOOD REQUIRED PER GM GAIN gm	AV. FOOD CONSUMED DAILY gm	AV. LIFE OF RATS <sup>1</sup> days
1	Egg	8	2.17 ± .11	2.50 ± .08	3.99 ± .20	8.65 ± .36	56
2	Peas	19	0.60 ± .04	0.95 ± .06	10.54 ± .68	6.36 ± .45	33
3	Wheat germ	12	2.37 ± .13	2.16 ± .09	4.73 ± .20	10.89 ± .12	56
4	Corn germ	12	2.36 ± .11	2.01 ± .06	5.01 ± .14	11.74 ± .13	56
5	Peas and 0.3% dl-methionine	19	1.88 ± .09	2.04 ± .06	4.89 ± .13	9.20 ± .39	56
6	50% Peas and 50% wheat germ	12	2.11 ± .11	1.96 ± .07	5.18 ± .20	10.74 ± .03	56
7	75% Peas and 25% wheat germ	7	1.77 ± .06	1.92 ± .05	5.23 ± .16	9.21 ± .02	56
8	87.5% Peas and 12.5% wheat germ	8	1.47 ± .04	1.69 ± .01	5.94 ± .15	8.75 ± .03	56
9	50% Peas and 50% corn germ	13	1.85 ± .11	1.97 ± .07	5.15 ± .19	9.33 ± .04	56
10	75% Peas and 25% corn germ	8	1.58 ± .05	1.75 ± .05	5.74 ± .14	9.02 ± .02	56
11	87.5% Peas and 12.5% corn germ	9	1.30 ± .08	1.51 ± .06	6.71 ± .24	8.61 ± .26	52

<sup>1</sup> Experimental period 56 days.

TABLE 3  
*Statistical comparisons of growth on various diets.*

LOT NO.	DIETS COMPARED	AVERAGE DAILY GAIN (GM)			AVERAGE GAIN PER GM PROTEIN (GM)		
		Mean difference	Standard error of difference	t <sup>1</sup>	Mean difference	Standard error of difference	t <sup>1</sup>
1-2	Egg vs. peas	1.57	.12	13.08	1.55	.10	15.50
1-3	Egg vs. wheat germ	0.20	.16	1.22	0.34	.12	2.82
1-4	Egg vs. corn germ	0.19	.15	1.22	0.49	.10	4.90
1-5	Egg vs. peas + 0.3% dl-methionine	0.29	.14	2.07	0.46	.11	4.18
3-4	Wheat germ vs. corn germ	0.01	.17	0.05	0.15	.11	1.38
2-5	Peas vs. peas + 0.3% dl-methionine	1.28	.10	12.80	1.09	.09	12.11
5-6	Peas + 0.3% dl-methionine vs. 50% peas + 50% wheat germ	0.23	.14	1.62	0.08	.09	0.87
5-7	Peas + 0.3% dl-methionine vs. 75% peas + 25% wheat germ	0.11	.10	1.66	0.12	.08	1.53
5-8	Peas + 0.3% dl-methionine vs. 87.5% peas + 12.5% wheat germ	0.41	.09	4.16	0.35	.07	4.85
5-9	Peas + 0.3% dl-methionine vs. 50% peas + 50% corn germ	0.03	.14	0.02	0.07	.09	0.76
5-10	Peas + 0.3% dl-methionine vs. 75% peas + 25% corn germ	0.30	.10	2.91	0.29	.08	3.71
5-11	Peas + 0.3% dl-methionine vs. 87.5% peas + 12.5% corn germ	0.58	.12	4.81	0.53	.08	6.24
6-9	50% Peas + 50% wheat germ vs. 50% peas + 50% corn germ	0.26	.15	1.67	0.01	.09	1.01
7-10	75% Peas + 25% wheat germ vs. 75% peas + 25% corn germ	0.19	.07	2.43	0.17	.07	2.40
8-11	87.5% Peas + 12.5% wheat germ vs. 87.5% peas + 12.5% corn germ	0.17	.08	1.90	0.18	.07	2.49

<sup>1</sup>A t value of 3.499 establishes the 1% point for the comparisons based on the smallest number degrees of freedom; therefore a value of 3.499 or larger is highly significant.

in rate of gain but were less efficient. In the case of corn germ this difference is statistically significant. A comparison of the pea diet data with those of whole egg emphasizes the superior quality of egg protein. As previously reported by Jones and Widness ('46) the growth response resulting from wheat germ protein was better than that from corn germ. In the experiments reported here, the *t* value does not indicate that the difference is significant.

It has already been reported from this laboratory (Lehrer et al., '47) that the addition of 0.3% dl-methionine improves both growth rate and efficiency of food utilization on a diet in which cooked peas supply the protein. Results from the diets in which the protein is supplied by mixtures of peas and either wheat or corn germ also show improved growth-promoting quality. Both rate of gain and efficiency were not different from those of the methionine diet when peas and either germ were used in equal proportions, or when the mixture contained 25% wheat germ and 75% peas. However, when the mixture was 25% corn germ and 75% peas, the efficiency of the mixed proteins was significantly lower than that for the peas and methionine diet. When the mixture was reduced to 12.5% of either cereal germ to 87.5% of peas neither one gave as good growth as the 0.3% methionine supplemented pea diet. This is true whether the measure used was average daily gain or average gain per gm of protein.

#### SUMMARY AND CONCLUSIONS

A study of the quality of the proteins of wheat germ and of corn germ as a supplement for peas has been made using the growth-promoting method with young rats.

When used as the sole source of protein both of the products studied compared favorably with whole egg protein in rate of gain but the corn germ was significantly less efficient.

As a supplement for whole peas, wheat germ in equal proportions or as 25% wheat germ to 75% cooked peas had as good a growth-promoting value as peas supplemented with 0.3% dl-methionine.

Corn germ, fed in equal proportions with peas, produced as good results as the methionine diet but was not as efficient as methionine when used as 25% of the mixture.

Neither product as 12.5% to 87.5% peas gave as good results as 0.3% dl-methionine added to peas.

Under the conditions of this experiment, the wheat germ product used was a better supplement for peas than the corn germ.

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# STUDIES ON THE CONCENTRATION OF SOME B-VITAMINS IN THE BLOOD OF NORMAL AND COBALT DEFICIENT SHEEP <sup>1, 2</sup>

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In a previous paper (Ray et al., '47), it was shown that injection of thiamine and possibly pyridoxine ameliorated the condition of cobalt deficiency in ruminants. It was suggested that in cobalt deficiency, the production of B-vitamins in the rumen of the deficient animal was disturbed and the symptoms of the deficiency may be due to avitaminosis. In order to check this point, it was decided to examine the concentration of some B-vitamins in the blood of normal and cobalt deficient sheep, as it was thought that if these vitamins were not being elaborated in sufficient quantity in the rumen of the deficient animal, their concentration in the blood would also fall. The results of this investigation are reported here.

## EXPERIMENTAL

The cobalt deficient sheep were all kept on a basal ration of cobalt deficient hay (Co content = 0.03 ppm) and a mixture of 80% corn and 20% corn gluten meal. In addition, the animals were offered iodized salt ad libitum. Some of these animals were born at the Experiment Station and had been

<sup>1</sup> Published with the approval of the Director of Wisconsin Agricultural Experiment Station.

<sup>2</sup> This work was supported in part by the Wisconsin Alumni Research Foundation.

on the cobalt deficient ration since their birth. The other animals of this group were purchased from farms where symptoms of cobalt deficiency had been prevalent and no cobalt therapy had been used. The normal animals were also kept on the identical ration except that they received cobaltized salt (12.7 gm  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  per 25 lbs. iodized salt). The composition of the basal ration was kept constant in both groups, as it had been found by Lardinois et al. ('44), that the composition of the feed has a great effect on the production of B-vitamins in the rumen. The animals were kept on these rations for 6 months to 1 year before they were examined. In the deficient group, blood was collected from only those animals which were just beginning to show deficiency symptoms, as evidenced by a slowing of the growth rate. At this stage, the food intake of the deficient animals was quite comparable to that of the normal animals. This policy was adopted as we did not want to have the results complicated by inanition, which is always seen in acute cobalt deficiency.

Oxalated blood was collected in the morning and was sent under ice to the laboratory where it was kept in the refrigerator until used. In no case was the blood more than 3 days old when examined. One ml of blood was mixed with 7 ml of acetate buffer (pH 4.5), 1 ml of takadiastase solution (20 mg per ml), and 1 ml of papain suspended in water (20 mg per ml) (Cheldelin et al., '42). A little benzene was added as a preservative to the mixture which was then incubated for 24 hours at 37°C. The mixture was then heated for one-half hour in flowing steam, cooled, and filtered. An attempt was made to estimate the vitamins in this filtrate by the recognized microbiological methods, but too low recovery and too much "drifting" was observed. A known volume of filtrate was therefore extracted with twice its volume of ether and the ethereal layer was discarded. The extraction was carried out once more and the aqueous layer was freed of ether by gentle heating on a hot plate and then made up to proper volume. This procedure eliminated entirely the "drifting" seen with

increased amount of unknown solutions. The recovery of added vitamins was also good, being mostly within 92–103%. Roberts and Snell's method ('46), using *L. casei* was used for the determination of riboflavin, pantothenic acid, folic acid, and niacin. For the estimation of total pyridoxine, the method of Atkin et al. ('43), as modified by Rabinowitz and Snell ('47), was employed, using *Saccharomyces Carlsbergensis* as the microorganism. For this last method, 1 ml of blood was mixed with 9 ml of 0.05 N HCl, autoclaved for 5 hours at 15 lbs. pressure, cooled, adjusted to pH 5.2, centrifuged, and the clear supernatant fluid used for analysis. With both *L. casei* and the yeast, an 18-hour incubation, followed by the turbidimetric measurement of the growth of microorganisms was used. In pyridoxine determination, the tubes were shaken continuously during the whole period of incubation.

The results of our findings are given in table 1.

TABLE 1

*Concentration of various vitamins in the blood of normal and cobalt-deficient sheep. All values are in  $\mu\text{g}$  per ml of blood, with means and standard deviations from the mean.*

VITAMIN	NORMAL	DEFICIENT
Riboflavin	$0.265 \pm 0.028$ (13) <sup>1</sup>	$0.235 \pm 0.035$ (12)
Folic acid	$0.046 \pm 0.008$ (8)	$0.044 \pm 0.007$ (6)
Pantothenic acid	$0.418 \pm 0.070$ (10)	$0.432 \pm 0.082$ (13)
Nicotinic acid	$12.14 \pm 1.81$ (8)	$9.53 \pm 2.99$ (6)
Vitamin B <sub>6</sub> group	$0.118 \pm 0.018$ (6)	$0.067 \pm 0.010$ (5)

<sup>1</sup> The figures in parentheses represent number of animals examined.

## DISCUSSION

No values for the riboflavin, folic acid, and vitamin B<sub>6</sub> contents for normal sheep blood are available in the literature. Our values for the riboflavin content for normal sheep are lower than those reported in normal animals of other species by Strong et al. ('41); but as these authors did not carry out any lipid separation from the blood extract, the values found by them might have been too high (Eckhardt et al., '41). Our

folic acid values are similar to those found for man, but higher than those of cattle or pigs (Schweigert and Pearson, '47). Our pantothenic acid values are much higher than those reported by Pearson ('41) for sheep. This may possibly be due to a difference in the rations of Pearson's animals, as it has been shown by Lardinois et al. ('46), that ration composition influences greatly the rumen synthesis of the vitamin-B complex. Our values for the nicotinic acid content of normal sheep blood are similar to those found by Pearson et al. ('40).

It may be seen that the concentrations of nicotinic acid and vitamin B<sub>6</sub> contents of the blood of deficient animals were definitely lower than those in normal sheep; there was a suggestion of a similar lowering in the case of riboflavin. It is plausible, therefore, that these 3 vitamins were not being produced in sufficient quantity in the rumen of the cobalt deficient sheep. The folic acid and pantothenic acid contents were, however, nearly the same in the 2 groups. This may indicate that the production of various members of vitamin-B complex may be brought about by different rumen microflora and cobalt may influence the production of some, but not of all the members.

#### SUMMARY

The concentrations of nicotinic acid and vitamin B<sub>6</sub> group in the blood of cobalt deficient sheep were found to be lower than those in the normal animals; there was a suggestion of a similar lowering in the case of riboflavin. The folic acid and pantothenic acid contents were not affected.

The relation of cobalt metabolism in ruminants to endogenous production of certain of the B-vitamins is discussed.

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## MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1948 Award of \$1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the Spring of 1948.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1 to December 31 the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1947 must be in the hands of the Nominating Committee by January 10, 1948. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

E. N. TODHUNTER  
*University of Alabama*  
*University, Alabama*

CHAIRMAN, MEAD JOHNSON NOMINATING COMMITTEE





# SOME OBSERVATIONS ON BEEF CATTLE AFFECTED WITH GENERALIZED EDEMA OR ANASARCA DUE TO VITAMIN A DEFICIENCY

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ONE FIGURE

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The fact that prolonged vitamin A deficiency in cattle may result in edema as characterized by swelling of the legs, shoulders and brisket, on the abdomen, in the hindquarters and elsewhere has been recognized for some time. This condition is often referred to as anasarca. Hastings ('41, '44), Cady ('42) and others have more recently called attention, in the popular press, to anasarca in fattening cattle apparently due to vitamin A deficiency in rations of which the grain consisted principally of yellow corn. Similar cases have been brought to the attention of the Bureau of Animal Industry when distillery slop and low-quality roughages have been fed to fattening cattle.

In 1941 our attention (Creech and Madsen, '42; Mohler, '42; and Madsen, '42) was called to a condition characterized by extensive edema of the subcutaneous adipose tissue and musculature of beef carcasses which was encountered by

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Federal meat inspectors at several meat packing establishments. In view of the unusually large number of cases of unrecognized origin (see table 1) the senior author was called to the Corn Belt area where a number of these cattle had been fed, to determine if a nutritional basis could be established for the disease since previous bacteriological studies had been entirely negative.

TABLE 1

*Beef carcasses condemned for anasarca by months from July, 1941 to December, 1946, inclusive.*

	YEAR						Monthly average
	1941	1942	1943	1944	1945	1946	
January	..	2	3	9	5	5	5
February	..	3	11	5	2	12	7
March	..	9	4	4	3	9	6
April	..	6	5	5	5	10	6
May	..	9	10	7	9	6	8
June	..	5	15	9	11	5	9
July	3	12	18	18	13	10	12
August	18	5	20	3	9	8	10
September	40	6	30	3	11	3	15
October	43	9	28	5	5	7	16
November	20	4	13	7	11	10	11
December	15	5	12	6	6	12	9
Yearly total	139	75	169	81	90	97	

The present paper reports statistics on the number of beef carcasses condemned for anasarca by the Federal meat inspectors during the years 1941-1946, and presents data on the occurrence, clinical appearance and history of field cases of anasarca as they were observed during the field trip to the Corn Belt in 1941. It also describes the experimental production of edema in vitamin A-deficient cattle and presents results of observations made on the blood chemistry in both field cases of anasarca and experimentally produced cases of this condition.

## CARCASSES CONDEMNED

The data given here on condemned carcasses were furnished through the courtesy of the Meat Inspection Division of the Bureau. The records show that 651 carcasses were condemned for anasarca by Federal meat inspectors from July, 1941 through December, 1946. The distribution of these cases on a monthly and yearly basis is given in table 1. Heavy losses were encountered in the last half of 1941. Fewer cases were condemned in 1942 but losses were heavy in 1943. About the same number were condemned in 1945 as in 1946. It appears likely that the years of relatively few numbers of anasarca cases are related to the prevailing light or short-time grain feeding of steers. From these limited data it seems that more cases appear in the latter half of the year. These statistics do not represent the total loss among slaughter animals due to anasarca since in cases in which the edema is localized the carcass may be retained, trimmed as found necessary under inspection and released.

## FIELD CASES OF ANASARCA

Statistics are not available on the number of cases of anasarca that develop under practical conditions. It is obvious, however, that more cases develop than are condemned at slaughter since some cases recover before slaughter because of timely changes in the ration, whereas others may die on the farm or become severely injured during convulsions while in transit. Seven farms having approximately 1200 cattle on feed were visited in the Corn Belt area by the senior author in company with Dr. John S. Koen and Dr. H. J. Classick, both veterinarians of the Bureau of Animal Industry on field assignments. Case reports from 2 of these farms are presented to illustrate (1) the development of vitamin A deficiency associated with anasarca under ordinary feed lot conditions and (2) a means of preventing serious losses by simple corrective feeding.

*Case 1.* Mr. G. started feeding 41 head of yearling Hereford steers, obtained from the West, in November, 1940. They were

started on feed with corn and fodder of the 1940 crop and about 1 pound per head per day of a mixture of equal parts molasses and alfalfa which was continued until June, 1941. When the hot weather came during the latter part of June and persisted through July and August the cattle "went off feed," but no edema was seen by the owner. At this time feeding alfalfa hay of the 1940 crop 3 times daily improved the appetite of the steers and they were soon brought back to full-feed. Early in September oat straw was substituted for the alfalfa hay. Straw feeding was continued until early in December when Dr. Koen visited the farm and suggested a change in roughage from oat straw to alfalfa on the basis of information he had received from the Bureau that anasarca may be due to vitamin A deficiency. At this time about 30 animals of the group had swollen legs and enlarged briskets. One steer could hardly walk and 1 animal had recently been shipped to Omaha and condemned for anasarca. We visited the farm on December 18, 1941, about 2 weeks after alfalfa feeding was started, and according to Dr. Koen improvement in the cattle was evident. Dr. Koen and Dr. Classick visited this farm again on January 21, 1942, and found so much improvement in the cattle that little or no edema could be observed. All of the cattle were sold about 1 week later, or approximately 8 weeks after alfalfa feeding was started, without evidence of anasarca.

*Case 2.* On this farm 135 yearling steers were started on feed in October, 1940. The animals were turned into a corn field where they had access to corn stalks and some blue-grass until January, 1941. At this time they were taken up to the feed lot and given Atlas sorghum silage at about 30 pounds per head daily and all the ground yellow corn (1940 crop) they would clean up. A small amount of soybean hay was also given until the supply was used, about June 1, 1941. At this time feeding of oat hay was started and corn silage was fed until August. After all the silage had been consumed, oat hay was continued as the only roughage. On November 6, 1941,

20 of the steers were shipped to Omaha, and of this group 1 was condemned for anasarca. A few more animals were sent to Chicago about the middle of November and 1 of these was also condemned for anasarca. Another steer became very lame and a veterinarian advised that the animal had probably sprained its leg, and that the steer should be killed and used for food. The steer was butchered but the carcass was declared unfit for food owing to generalized edema.

When we visited the farm on December 19, 1941, several animals showed marked symptoms of vitamin A deficiency. One animal was blind and had swollen legs and brisket. This animal was down and died the following day. Two other steers were also markedly affected, 1 was blind and another had severe edema of the legs, brisket and shoulders.

Twelve days previous to our visit Dr. Koen had seen these cattle and suggested feeding alfalfa as in case 1. This had resulted in a marked improvement in appetite and general appearance of the steers. The foreman commented that the cattle could get up and go to the feed bunks with greater ease since they had been receiving alfalfa, and that the lameness which had been previously noted had now practically disappeared. The foreman also characterized as "fish-eyes" the peculiar protruding eyes of several of the cattle, a condition which is typical of vitamin A-deficiency blindness.

The findings presented in these 2 cases were typical of conditions noted on other farms where cases of anasarca were seen. In some instances other symptoms of vitamin A deficiency such as diarrhea, constipation, nightblindness, stiff gait and convulsions were prominent. In 1 instance impaired vision in the cattle was not suspected until difficulty was encountered in loading the cattle on a truck. On this farm the vision of an apparently blind steer improved remarkably after alfalfa hay had been added to the ration in place of oat hay. In some feed lots anasarca developed in cattle fed stored corn, but in these cases a low-carotene roughage was also used.

## EXPERIMENTAL CASES OF ANASARCA

Experimental cases of anasarca were produced by feeding either a carotene-deficient ration (diet 1) or one containing a large amount of yellow corn (diet 2), the roughage in both cases being supplied by oat straw. The concentrate portion of diet 1 had the following percentage composition: dried beet pulp, 45; cracked white corn, 40; linseed meal, 7; soybean meal, 7; steamed bonemeal, 0.5; and salt, 0.5. The concentrate portion of diet 2 consisted, in per cent, of cracked yellow corn, 80; dried beet pulp, 8.5; linseed meal, 10; steamed bonemeal, 1; and salt, 0.5. Vitamin A-deficiency edema was readily produced when cattle were restricted to either of these rations for several months. In a number of instances, after the appearance of anasarca, the diet was supplemented with 1 of several sources of carotene and vitamin A such as alfalfa leaf meal, carotene concentrate, crystalline carotene, cod-liver oil and distilled vitamin A concentrate in natural ester form. In all such cases, anasarca, as well as other symptoms of vitamin A deficiency except permanent blindness, was entirely relieved with the treatment.

A complete history is given for only 1 case (steer 411) in which the course of depletion and the development of deficiency symptoms were quite characteristic of all the other cases. Since a significant part of the story is believed to be told by the results of the blood analyses which were made at approximately monthly intervals on a number of these edema cases, these data are included in the history, although the general subject of blood analyses in these cattle is treated in a later section of this paper.

Steer 411 on which the case history of experimental edema is based was fed individually on diet 2 (80% yellow corn). This animal was a purebred Shorthorn approximately 7 months old weighing 613 pounds at the beginning of the experiment. Feed consumption, live-weight gain, together with the appearance of progressive symptoms of convulsions, diarrhea, blindness and edema are indicated in table 2 and illustrated in figure 1. Attention is called to the fact that

TABLE 2

Summary of observations on steer no. 411 while receiving an 80% yellow corn grain ration.

OBSERVATION	DAYS OF EXPERIMENT										
	27	58	90	117	147	177	209	238	271	289	300
Condition of animal	Normal	Normal	Normal	Normal	Normal	Con-				Edema	
Body weight, lbs.	635	675	712	765	827	vulsions	921	970	1033	1056	1062
Average daily gain for period, lbs.	1.41	1.29	1.13	1.96	2.07	873	1.50	1.69	1.91	1.98	0.55
Daily feed consumption:						1,53					
Grain mixture, lbs.	7.7	8.9	10.8	11.9	12.1	13.9	14.6	15.1	15.7	16.0	17.9
Out straw, lbs.	2.2	1.6	1.8	2.2	2.2	2.0	2.0	1.2	1.0	1.7	1.3
Plasma nitrogen fractions:											
Total plasma nitrogen, gm/100 ml	1.019	0.903	1.046	1.032	1.107	1.105	1.007	1.051	1.019	1.071	1.089
Nonprotein nitrogen, gm/100 ml	0.022	0.041	0.048	0.039	0.061	0.041	0.028	0.029	0.025	0.022	0.024
Fibrinogen nitrogen, gm/100 ml	0.052	0.060	0.088	0.063	0.074	0.060	0.055	0.075	0.064	0.133	0.133
Proglobulin nitrogen, gm/100 ml	0.089	0.081	0.082	0.081	0.106	0.076	0.119	0.096	0.081	0.083	0.085
Pseudoglobulin nitrogen, gm/100 ml	0.283	0.288	0.308	0.320	0.320	0.310	0.270	0.309	0.319	0.362	0.363
Total globulin nitrogen, gm/100 ml	0.424	0.429	0.478	0.464	0.500	0.446	0.444	0.480	0.464	0.578	0.590
Albumin nitrogen, gm/100 ml	0.573	0.523	0.520	0.529	0.546	0.618	0.535	0.542	0.530	0.471	0.457
A/G ratio	1.35	1.22	1.09	1.14	1.09	1.39	1.20	1.13	1.14	0.81	0.77
Plasma:											
Carotene, $\mu$ g/100 ml	86	35	32	33	46	39	38	40	46	....	27
Vitamin A, $\mu$ g/100 ml	25	16	23	11	13	13	9	10	7	....	8
Vitamin C, $\mu$ g/100 ml	0.41	....	....	0.56	0.54	0.40	0.39	0.43	0.34	....	0.31
Serum:											
Calcium, mg/100 ml	....	....	13.1	10.6	11.0	12.0	10.9	10.2	11.1	....	11.5
Magnesium, mg/100 ml	2.8	....	2.6	2.6	2.4	2.5	2.4	2.3	2.3	....	2.7
Inorganic phosphorus, mg/100 ml	....	7.8	8.1	8.2	7.6	7.7	6.1	6.9	7.2	5.8	6.3
Phosphatase activity (Bodansky units)	....	4.5	3.1	4.9	4.6	4.2	3.8	4.6	4.1	4.4	3.3

<sup>1</sup> Steer died during a convulsion on the 343rd day.<sup>2</sup> Beginning stages of rough hair coat, slobbering and slight edema.<sup>3</sup> Edema increasing rapidly; convulsions more numerous. Ascorbic acid therapy started 303rd day.

there was an increase in grain consumed up to the three hundredth day, but that feed consumption and rate of gain decreased after this time. No blood analyses were made after the three hundred and twenty-eighth day. Typical changes in the plasma protein fractions were observed at about the same time that edema appeared. Plasma carotene, vitamin A and



Fig.1 .These photographs illustrate symptoms of vitamin A deficiency in steer 411 after 309 days on diet 2. A. The steer is blind and has a rough, dry hair coat, but has made fairly good gains in weight. B. The hind legs are swollen due to edema. C. Rapid respiration was common and even panting occurred on slight exertion in hot weather. Note slobbering. D. Convulsions occurred frequently. The head is drawn back typically. Note left horn fracture (C) received during previous convulsions.



vitamin C decreased in the usual manner, but there were minor fluctuations in these during the experiment. Serum calcium, inorganic phosphorus and phosphatase activity tended to decrease but the changes were not marked until after about the three hundredth day at which time the food intake also decreased.

The highest average grain consumption for steer 411 was recorded for the period between the two hundred and eighty-ninth and three hundredth days. An average of 17.9 pounds of grain mixture or 14.3 pounds of yellow corn was eaten daily at this time. On analysis the corn was found to contain 2.1  $\mu$ g of crude carotene per gram and on biological assay with rats a value of 1.6 International Units (I.U.) of vitamin A per gram was obtained. At the above level of yellow corn consumption, and using the assay figures just mentioned, the steer received 13.6 mg of crude carotene daily, or 10,378 I.U. of vitamin A. This amount of carotene from yellow corn was insufficient to alter the course of vitamin A depletion. Feed consumption declined, edema increased and the animal died in convulsion on the three hundred and forty-third day of the experiment. Another steer on the same ration died in convulsion on the two hundred and forty-ninth day of the experiment. In this case edema appeared about the two hundred and thirteenth day, and some impairment of day vision was evident before death.

These findings add further evidence to the field observations that vitamin A-deficiency symptoms together with edema develop in fattening cattle when their ration is made up of yellow corn, a protein supplement and a low-carotene roughage.

#### BLOOD ANALYSES

Before attention was directed to the field cases of anasarca occurring in the Corn Belt, some data were already available on the composition of blood from a number of experimental animals both under conditions of apparently normal health and also at various stages of vitamin A deficiency which developed during prolonged use of diet 1. These data seemed

to indicate that there were certain characteristic changes in the composition of the blood, particularly with respect to the protein fractions, approximately coincident with the development of edema in these vitamin A-deficient animals. Analyses of blood obtained from some field cases of anasarca were undertaken primarily for the purpose of further characterization of the general physiological condition of the animals and also for the further identification of this condition with the edema occurring in the experimental animals depleted of vitamin A. Results of analyses of blood from the field cases seemed to indicate that changes had occurred here similar to those in the experimental edematous animals. In view of the feeding practices under which the anasarca cases observed had developed in the Corn Belt, blood studies were also made on animals which were fed diet 2 containing 80% yellow corn.

Blood samples for analysis were drawn from a jugular vein. For the preparation of plasma samples, exactly 30 mg of neutral potassium oxalate were used as anticoagulant for each 10 ml of blood. Plasma proteins were fractioned by Howe's ('21) micro-method with the variation later recommended by him ('23) that 0.75 molar sodium sulfate be used for the precipitation of fibrinogen in plasma instead of calcium chloride. All nitrogen determinations were made on aliquots of the filtrates by a micro-Kjeldahl method. The spectrophotometric method previously described by Madsen and Davis ('38) and Davis and Madsen ('41) was used for determination of plasma carotene and vitamin A. Plasma vitamin C was determined by the method of Mindlin and Butler ('38). Serum calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method as described by Hawk and Bergeim ('37); serum magnesium was precipitated according to the method used by Godden ('37) and determined as phosphorus by the method of Bodansky ('32). Bodansky's ('33) methods were also used for the determination of serum inorganic phosphorus and serum phosphatase activity.

## RESULTS

The results of analyses on cases of experimental edema produced in 7 beef animals have been averaged and paired with the average of observations made on these same animals when they were normal. The averages are presented in table 3 together with the average results of blood analyses made on 5 field cases of anasarca.

It is obvious from the figures presented in table 3 that the plasma protein fractions in the experimental animals with edema differ significantly from those in the same animals

TABLE 3

*Summary of results of blood analyses from field and experimental cases of anasarca and normal beef animals.*

CONSTITUENTS	AVERAGE OF 5 FIELD CASES OF ANASARCA	EXPERIMENTAL CASES		
		Averages of 7 with edema	Averages of 7 when normal	Calculated odds — edematous vs normal <sup>1</sup>
Nitrogen fractions in plasma:				
Total plasma nitrogen, gm/100 ml	1.263	1.150	1.076	104
Non-protein nitrogen, gm/100 ml	0.038	0.036	0.037	
Fibrinogen nitrogen, gm/100 ml	0.151	0.150	0.061	191
Euglobulin nitrogen, gm/100 ml	0.166	0.180	0.136	51
Pseudoglobulin nitrogen, gm/100 ml	0.494	0.384	0.318	1999
Total globulin nitrogen, gm/100 ml (including fibrinogen)	0.811	0.714	0.515	2499
Albumin nitrogen, gm/100 ml	0.414	0.400	0.524	587
A/G ratio	0.51	0.56	1.02	
Vitamins in plasma:				
Carotene, µg/100 ml	31	21	67	26
Vitamin A, µg/100 ml	8	7	23	9999
Vitamin C, mg/100 ml		0.25 <sup>2</sup>	0.37 <sup>2</sup>	13
Constituents in serum:				
Calcium, mg/100 ml		10.01	11.00	160
Magnesium, mg/100 ml		2.63	2.72	
Inorganic phosphorus, mg/100 ml		5.60	6.74	40
Phosphatase activity (Bodansky units)		2.28	4.24	232

<sup>1</sup> Student's method used for estimating significance between means of paired observations in experimental cases of anasarca and corresponding normal control values.

<sup>2</sup> Average for only 4 animals.

when normal. Attention is called to the increases in fibrinogen and total globulin and to the decrease in albumin in the animals with edema.

Low values for plasma carotene and vitamin A were observed in all cases of anasarca. Calculated odds for estimating the significance of differences in the pairs of average values for plasma carotene and for vitamin A were greater for vitamin A than for carotene. This may be explained by the uniformly low values for vitamin A and carotene in all cases of anasarca as contrasted with uniformly high values for vitamin A and high, but variable, values for carotene in normal animals. The carotene intake of the normal cattle was not standardized, but in all cases was above a deficiency level. In the field cases of anasarca the average blood carotene and vitamin A were higher than in the experimental cases, a condition which is probably due to the fact that alfalfa hay feeding had already been started in some of these animals when blood samples were taken.

Average content of vitamin C in plasma was found to be lower while edema was present than when it was absent in the same animals. The significance of the difference in the 2 averages is shown by the calculated odds not to be great, however, probably because data for this comparison were available from only 4 pairs of observations and because the degree of vitamin A deficiency was not the same in all the animals when the observations were made. In the 4 cases observed there was always a decrease in plasma vitamin C as the deficiency of vitamin A progressed but the drop was not uniform in all cases. This decrease in plasma vitamin C in cases of vitamin A deficiency was first demonstrated by Phillips and coworkers ('38).

While serum calcium, magnesium and inorganic phosphorus tended to decrease slightly from the levels observed when the same animals were normal, the values still remained well within the normal range. There was, however, a decrease from normal in phosphatase activity of the serum in all cases of experimental anasarca studied.

## DISCUSSION

Losses due to anasarca associated with vitamin A deficiency are economically important. Nevertheless they can be prevented by proper feeding. Supplying fattening cattle with a small amount of well-cured alfalfa hay successfully avoided serious losses in 2 instances cited. Any other well-cured hay having a good green color, well-made silage, green pasture or a marine oil such as cod-liver oil would no doubt have accomplished the same purpose. The prevention of anasarca or of other symptoms of vitamin A deficiency by the use of proper feeds does not necessarily discourage the highly economical practice of feeding low-carotene roughages such as ordinary cereal hay, straw and corn stover, etc. When these roughages are used, however, it is necessary to supply a supplementary ration of well-cured green hay to avoid possible vitamin A depletion in the latter part of the feeding period.

Opinions have been expressed in the literature and have been prevalent among some cattle feeders that anasarca is caused by feeding stored corn. In both field and experimental cases of anasarca presented in this paper, however, yellow corn of the current season's crop failed to prevent the development of either anasarca or other symptoms of vitamin A deficiency. This observation confirms the report by Hastings ('44). The real error in practical feeding appears to be not in the use of old corn but in the exclusive use of low-carotene roughages such as oat hay and straw and the failure to supplement these low-carotene roughages with green hay less than 1 year of age. Average yellow corn usually contains no more and often less carotene than a low grade hay. Corn should not be depended upon to supply enough carotene to satisfy the vitamin A needs of cattle.

The clinical symptoms and blood findings presented suggest that the field cases and experimental cases of anasarca probably arose from the same condition. All cases of anasarca observed evidenced low levels of blood carotene and vitamin A and, in addition, showed the same changes in blood protein

fractions. These changes consisted essentially of an increased level of total globulins, due principally to increased fibrinogen and, to a lesser degree, to increases in the euglobulin and pseudoglobulin fractions. At the same time there was a definite, although not in every case large, decrease in plasma albumin. The total protein was usually slightly elevated when edema was present.

The relation of the altered protein picture to vitamin A deficiency is not apparent. Increases in fibrinogen in conditions in which destruction and inflammation of tissues occur and increases in other globulin fractions, either with or without increased fibrinogen, in cases of chronic infections, are usually accompanied by some reduction in albumin concentration, an effect presumably secondary to the increase in globulin. Since a reduction in blood albumin is a primary factor in some types of edema, the question arises as to the relation between the lowered albumin and the development of edema in these cattle. The concept of an increased tendency toward transudation and accumulation of fluid in the interstitial tissues with a lowering of the plasma colloid osmotic pressure is a familiar one. In this relation, the concentration of albumin is of greater significance than that of globulins, since the albumin exerts about 2.4 times as great an osmotic pressure as the globulins. It is estimated that at the so-called critical levels for albumin nitrogen and for total plasma nitrogen in the human (i.e., levels below which edema usually appears), the plasma colloid osmotic pressure is reduced to roughly two-thirds of the normal level (Moore and Van Slyke, '30).

If it is assumed that approximately similar relative levels for albumin and for total protein are critical for cattle, it becomes apparent that the average reduction in albumin observed in the anasarca animals is not great enough to account for the appearance of edema, especially in view of the increase in total protein. Values for colloid osmotic pressure calculated by application of the formula of Wells, Youmans and Miller ('33) to data obtained on concentration of proteins in

serum of these animals (unpublished data), also suggest that increases in globulins are great enough to compensate in large measure for the reduction in albumin. It is believed, therefore, that the occurrence of edema in these animals is not entirely related to a lowering of the colloid osmotic pressure of the plasma brought about by a lowering of the albumin concentration.

#### SUMMARY

Statistics are presented showing that 651 beef carcasses were condemned for generalized edema or anasarca by Federal meat inspectors during the period of July, 1941 to December, 1946, inclusive. Other symptoms of vitamin A deficiency were found to be prevalent among cattle with anasarca in the Corn Belt area. This edematous condition was observed to occur in cattle after a long fattening period in dry lot when fed either stored or new corn in combination with a roughage of low carotene content such as oat hay or straw. Alfalfa hay was highly effective in curing the condition. It is concluded, therefore, that the major dietary error in the production of this deficiency disease is the exclusive use of low-carotene roughages throughout the feeding period rather than the use of old yellow corn.

Cases of anasarca were readily produced experimentally by feeding a carotene-deficient ration or a grain ration containing 80% of new-crop yellow corn together with oat straw as roughage.

Results of blood studies made on field and experimental cases of anasarca produced by vitamin A deficiency indicate a marked similarity in the 2 conditions. Affected animals showed: (1) deficiency levels of blood plasma vitamin A and carotene and a decrease in plasma vitamin C as the vitamin A deficiency progressed, (2) an increase in total plasma globulin due usually to a marked increase in plasma fibrinogen with smaller increases in the other globulin fractions, (3) a decrease in plasma albumin, and (4) an increase in total plasma nitrogen.

In the serum of affected cattle, calcium and inorganic phosphorus decreased slightly; phosphatase activity was reduced while magnesium was relatively unchanged.

It is pointed out that the edema of vitamin A deficiency in cattle appears not to be entirely related to a lowered colloid osmotic pressure of the plasma through reduction in the albumin, since the increases in other protein fractions are estimated to compensate largely in this respect for the decrease in albumin.

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# THE FOLIC ACID REQUIREMENT OF TURKEY POULTS ON A PURIFIED DIET<sup>1, 2, 3</sup>

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## TWO FIGURES

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The deficiency syndrome in turkey poults, caused by a lack of vitamin B<sub>12</sub>, now known to be a form of pteroylglutamic acid, was first described by Richardson, Hogan and Kempster ('45). Growth failure, cervical paralysis, and lowered red cell volume were the principal deficiency signs. Folic acid (pteroylglutamic acid) deficiency in turkey poults has more recently been described by Jukes and associates ('47).

The present paper is concerned with the determination of the folic acid level necessary for full growth, normal hemoglobin production and normal feathering of the turkey poult fed purified diets which differ in their protein content.

## EXPERIMENTAL

One hundred and two turkey poults which had been fed a starting mash for the first 8 days of life, were distributed in 11 groups according to weight and color type, so that each

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group contained 10 birds, except that groups 8 and 9, which received no folic acid, were allotted only 6 poultts each. The 4 color types which comprised the hatch were distributed so that each group contained 5 or 6 poultts of the Jersey buff color type and 1 or 2 each of the black, barred, or white types, to make a total of 10.

The 2 purified diets differed in their contents of casein, gelatin and starch but contained the same quantities in grams per 100 gm of diet, of the following ingredients: sucrose, 10; corn oil, 10; mineral salts<sup>5</sup>, 5;  $\text{CaHPO}_4$ , 2.0;  $\text{CaCO}_3$ , 0.4; cod liver oil (2,000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per gram), 0.7; choline chloride 0.2; inositol, 0.1; and para-aminobenzoic acid, 0.1. The following quantities of additional vitamins were provided per 100 gm of diet: thiamine, 1 mg; pyridoxine, 1 mg; riboflavin, 2 mg; calcium pantothenate, 2 mg; nicotinic acid, 5 mg; 2-methyl-1, 4 naphthoquinone, 2.5 mg; mixed tocopherols, 2.5 mg; and biotin, 10  $\mu\text{g}$ .

In an earlier experiment, poultts which consumed a purified diet in which 43% protein was supplied by casein (37%) and gelatin (10.6%) grew better than those on a starting mash, made of commonly used ingredients, containing 25% protein. For the present experiment these same diets were used, and in addition a purified diet containing 25% protein supplied by casein and gelatin, in the same ratio as that used for the diet containing 43% protein, so that a comparison could be made of the results, at the same protein level, on the purified diet and on the starting mash.

Diet A (25% protein) was prepared by adding 21.4 gm of casein (alcohol extracted), 6.1 gm of gelatin, and 44 gm of starch to the above ingredients to make 100 gm.

<sup>5</sup> The salt mixture, devised by A. G. Hogan (personal communication), had the following composition and was modified only by doubling the quantity of manganese. The quantities are expressed in grams.  $\text{CaCO}_3$ , 267;  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , 416;  $\text{MgCO}_3$ , 25;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 30;  $\text{NaCl}$ , 127;  $\text{KCl}$ , 21;  $\text{FePO}_4 \cdot 4\text{H}_2\text{O}$ , 20;  $\text{KH}_2\text{PO}_4$ , 315;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 50;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1.6;  $\text{Al}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ , 0.24;  $\text{KI}$ , 0.6;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.06;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05; and  $\text{NaF}$ , 1.0.

Diet B (43% protein) was prepared in the same manner by the addition of 36.7 gm of casein (alcohol extracted), 10.5 gm of gelatin, and 24.3 gm of starch.

Diet C (25% protein) was an open formula, commercial starting mash of the following percentage composition: wheat bran, 8; wheat standard middlings, 7; crushed wheat, 11; yellow corn meal, 15.5; low fiber oats, 7; alfalfa meal, 7.5; soybean oil meal (41%), 24; meat scrap (55% protein), 4; fish meal, 4; brewers' yeast, 3; riboflavin supplement, 0.4; dried whey, 5; ground limestone, 2; dicalcium phosphate, 1; iodized salt, 0.5; and "D" activated animal sterol, 0.1. Manganese sulfate was included in the ration so that each 100 gm contained 0.018 gm. Dried whey was fortified with riboflavin to contain a minimum of 5 mg per 100 gm. The D-activated animal sterol provided 200 A.O.A.C. chick units of vitamin D per 100 gm of ration.

Synthetic folic acid was added as an alcoholic, buffered solution containing 0.5 mg per ml, at the feeding levels shown in the tables.

## RESULTS AND DISCUSSION

### *Growth*

After 40 days (48 days of age) and after 56 days (64 days of age) on experiment, the poults receiving 43% protein, diet B, were significantly heavier than those on diet A, containing 25% protein, at all levels of folic acid supplementation (table 1). For each protein level, the weights at 40 days showed a tendency to be greater as the quantity of folic acid in the ration was increased, except that groups 14 and 17 showed lower body weights than those of the groups receiving the next lowest level of folic acid. At this time the poults fed the starting mash, group 18, weighed less even than those on diet B with 0.5 mg of folic acid.

After 56 days on experiment, too few birds remained in groups 10, 11 and 13 to permit their inclusion in a comparison of body weights. Those consuming diet B and 1.5 and 2.0 mg of folic acid per kg of feed were significantly heavier than

those on the corresponding levels of folic acid and being fed diet A (25% protein). This difference was undoubtedly due to the difference in protein levels. At this time, the poult fed diet C (starting mash) were slightly but not significantly heavier than the group on diet B and a 2.0 mg level of folic acid. Actually, only 2 poult on the starting mash were heavier than those receiving diet B.

TABLE 1  
*Growth of turkey poult on graded levels of folic acid.*

GROUP NO.	FOLIC ACID	INITIAL WEIGHT 8 DAYS	WEIGHT, 40 DAYS OF EXPERIMENT; 48 DAYS OF AGE	WEIGHT, 56 DAYS OF EXPERIMENT; 64 DAYS OF AGE
	mg/kg	gm	gm	gm
Diet A. 25% protein				
8	0.0	68 ( 6) <sup>1</sup>	( 0)	( 0)
10	0.5	74 (10)	418 ( 8)	(490) <sup>2</sup> (1)
12	1.0	74 (10)	449 (10)	608 (10)
14	1.5	72 (10)	422 (10)	643 (10)
16	2.0	74 (10)	469 (10)	682 (10)
Diet B. 43% protein				
9	0.0	71 ( 6)	( 0)	( 0)
11	0.5	74 (10)	579 ( 6)	(600) ( 1)
13	1.0	71 (10)	659 ( 7)	(648) ( 4)
15	1.5	72 (10)	725 ( 8)	918 ( 8)
17	2.0	73 (10)	684 ( 8)	974 ( 7)
Diet C. 25% protein in starting mash				
18	Starting mash	74 (10)	562 ( 9)	1017 ( 9)

<sup>1</sup> Poult per group.

<sup>2</sup> Insufficient number of poult for comparison.

The greater gains noted, after 40 days on the experimental diet in the case of all of the groups fed 43% protein and folic acid supplements, in contrast with the gain on the starting mash, may have been due to the fact that the pattern and quantity of amino acids presented by the casein and gelatin of diet B met more adequately the requirements for early rapid growth than did the ingredients of diet C, the starting mash. At 56 days these requirements may have been less,

or the folic acid supplements, except for the 2.0 mg level, may have been inadequate for the heavier birds.

### *Cervical paralysis*

Observations of the development and incidence of cervical paralysis are shown in table 2. Paralysis occurred in all groups

TABLE 2

*Development of cervical paralysis and mortality in turkey poults.*

GROUP NO.	INITIAL NO. OF BIRDS	FOLIO ACID	AVERAGE NO. DAYS TO PARALYSIS	DAYS TO FIRST CASE	DAYS TO LAST CASE	AVERAGE NO. DAYS TO DEATH	MORTALITY	
							Cervical paralysis no.	Other causes
mg/kg								
Diet A. 25% protein								
8	6	0.0	37	36	37	38	5	1 nonpathological
10	10	0.5	44	33	56	45	9	1 nonpathological
12	10	1.0	62	59	64	63	7	
14	10	1.5	59	59	59	62	2	1 nonpathological
								1 perosis
16	10	2.0	0	0	0	0	0	2 nonpathological
Diet B. 43% protein								
9	6	0.0	27	21	30	29	6	
11	10	0.5	41	32	56	43	10	
13	10	1.0	50	34	55	51	5	1 nonpathological
								2 perosis
15	10	1.5	52	54	68	53	4	2 nonpathological
								1 perosis
17	10	2.0	0	0	0	0	0	2 nonpathological
Diet C. 25% protein in starting mash								
18	10	Starting mash	0	0	0	0	0	1 nonpathological

on the purified diet at both protein levels, except in those which received 2.0 mg of folic acid, the incidence decreasing with an increase in the folic acid level.

On diet A (25% protein), without folic acid, 5 of the 6 poults in the group died following the onset of cervical paralysis and the other member died of a nonpathological cause 2 days

after the start of the experiment. In this group the first case of paralysis was noted 36 days after the start of the experiment and all 5 poultts that developed paralysis were dead by the thirty-eighth day. Among the poultts of group 9, consuming diet B (43% protein) without a folic acid supplement, the first case appeared after 21 days of experiment and the last after 30 days, the average number of days to death being 29. The 6 poultts of this group all died of cervical paralysis. It is noteworthy that on the 43% protein level, paralysis appeared 15 days earlier and the average number of days until death was 9 less than that for the group consuming diet A (25% protein). This difference in the onset of paralysis may be due to the more rapid growth at the higher protein level.

On both protein levels, when the folic acid supplied was 0.5 mg, all of the birds died of cervical paralysis except 1 on diet A which died of a non-pathological cause 12 days before the first case of paralysis occurred. In these groups and in those receiving 1.0 and 1.5 mg of folic acid paralysis appeared earlier and the average number of days to death was less at the 43% protein level than at the 25% level.

Although at the 1.5 mg level of folic acid, 2 cases of cervical paralysis appeared in the group which received diet A (25% protein) and 4 in the group fed diet B (43% protein), an increase of the folic acid supplement by 0.5 mg per kg of feed, so that the supplement was 2.0 mg, gave complete protection against the development of paralysis. No cases of paralysis appeared on diet C, the starting mash.

### *Feathering*

Throughout the experiment the feathering of all poultts, except those receiving 2.0 mg of folic acid and diet B (43% protein), was subnormal as compared with that of the poultts on the starting mash. The feathering departed from the normal in that breast feathers did not fit the body, and the tail feathers were sparse and broken. The wing feathers were



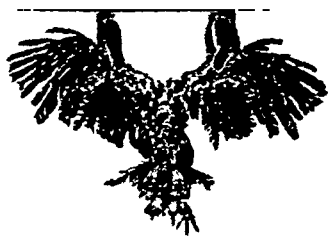
ragged and broken and did not lie close to the body. The feathering of poult on diet B (43% protein) and 2.0 mg of folic acid was indistinguishable from that of poult fed the starting mash, except that a few individuals showed some broken feathers which may have been due to cage damage and a tendency for the wings to droop. The light tan color of the Jersey buff poult was true to type for all groups



25 % PR. 1.0 MG. FA.



43 % PR. 1.0 MG. FA.



25 % PR. 1.5 MG. FA.



43 % PR. 1.5 MG. FA.

Fig. 1 Feathering of poult in relation to protein (PR.) and folic acid (FA.) levels, per kg of ration.

except those which received 43% protein and 2.0 mg of folic acid, in which case the color was definitely darker. The darker brown color of the plumage was also noted to an even more marked degree in an earlier experiment.

Wing and tail feathers of typical subjects after 56 days on experiment are shown in figures 1 and 2. Using the feathering of the poult fed the starting mash as normal, it is apparent that only for the poult on the purified diet B, which

provided 43% protein and 2.0 mg of folic acid, was the wing and tail feathering the same as that of the poult considered to be normal.



25 % PR. 2.0 MG. FA.



43 % PR. 2.0 MG. FA.



43 % PR. 2.0 MG. FA.



25 % PR. ST. MASH

Fig. 2 Feathering of poult in relation to protein (PR.) and folic acid (FA.) levels, per kg of ration and a commercial turkey starting mash (ST.MASH).

### *Hematology*

Hemoglobin values were obtained from samples drawn from the heart at intervals and as soon as signs of cervical paralysis appeared, the method of Schultze and Elvehjem ('34) being used. Readings were made with a Pfaltz and Bauer photo-electric colorimeter and values per gram of hemoglobin per 100 ml blood were obtained from a standard curve for which dilutions of an acid hematin standard solution were used. The readings were all taken with the diaphragm at 10 and using a 5200-5300 Å filter. In table 3 are listed the group averages of the hemoglobin determinations. The group receiving

the turkey starting mash has been taken as normal in considering the relative hemoglobin values.

It is evident that the hemoglobin values of the blood do not increase in direct proportion to the folic acid content of the feed. For none of the poults which survived 72 days of experiment on the purified ration at the 25% protein level, was the hemoglobin value as high as that obtained, at the corresponding folic acid level, when 43% protein was fed, indicating that the quantity of protein in the ration influences the hemoglobin content of the blood. On the purified ration containing 43% protein, only the group which received 2.0 mg

TABLE 3

*Hemoglobin values for turkey poults at various levels of folic acid supplementation.*

GROUP NO.	FOLIC ACID	PROTEIN	AVERAGE Hb OF POULTS WHICH DEVELOPED PARALYSIS	AVERAGE Hb OF SURVIVORS (72 DAYS)
	mg/kg	%	gm/100 ml	gm/100 ml
8	0.0	25	6.7	
10	0.5	25	6.6	
12	1.0	25	7.3	9.8
14	1.5	25	6.1	8.8
16	2.0	25		10.5
9	0.0	43	6.9	
11	0.5	43	5.7	..
13	1.0	43	6.4	12.3
15	1.5	43	6.2	11.6
17	2.0	43		12.7
18	Starting mash	25		13.0

of folic acid had a hemoglobin value essentially of the same order as that shown by the poults on the starting mash. The values for the blood of the paralytics, however, are essentially the same at both protein levels except that some lower values occurred in the groups on 43% protein. Jukes et al. ('47), on the other hand, have reported a hemoglobin level of 10 gm at 4 weeks on a diet containing approximately 25% protein.

#### *General appearance*

Frequent examination of the eyes, the skin of the feet, and the head did not reveal any abnormalities. Except for 4 cases

of perosis the posture of the birds was normal. All of the birds on the purified diet had pendulous crops in an earlier experiment, but only a few cases were observed in the present trial.

#### GENERAL DISCUSSION

It is evident that, of the 2 percentages of protein used in the purified diets, 25% and 43%, the higher at each folic acid level promoted better growth and feather formation and higher hemoglobin. Luckey and coworkers ('46) have concluded that for the chick a definite requirement for folic acid cannot be established because the response to a given quantity depends upon the type of ration used. The diet used by these investigators, which approached most closely the composition of purified diet A (25% protein) of the present experiment, promoted at 4 weeks of age, when the folic acid level was 1.0 mg per kilo, growth and hemoglobin formation inferior to that noted when corn meal replaced starch and contributed part of the protein, and 0.5 mg of folic acid was supplied per kilogram. The corn meal diet used by these investigators is the closest approach to the starting mash used in our experiment. These workers reported normal feathering in the chick when a purified type of diet was fed containing approximately 25% protein (by calculation) from casein and gelatin, and 0.5 mg of folic acid per kilogram of diet. When the protein of the diet, approximately 40% (by calculation) was supplied by casein only, subnormal feathering was obtained even when 3.0 mg of folic acid per kilogram of diet was provided. These observations and the difference in the feathering of poults at 2 protein levels in the present experiment suggest that, as long as other requirements are met by known factors, it may be the quantity of amino acids essential for feathering, that is responsible for normal feathering rather than a specific feathering factor.

Robertson et al. ('46) report that for the chick, at 6 weeks of age, approximately 0.25 mg of folic acid per kilogram of diet is necessary for survival; 0.45 mg for growth; 0.35 mg for

hemoglobin formation and not less than 0.55 mg for feathering. The diet used by these investigators was of the purified type, containing 30% protein (by calculation) plus a factor S concentrate equivalent to 5% yeast. The turkey poult, according to the results reported in the present paper, requires at least 4 times the quantity of folic acid for the several functions listed as does the chick. The higher requirement is in agreement with the generally accepted observation that the turkey poult has a greater need for certain nutrients as compared with the chick.

#### SUMMARY AND CONCLUSIONS

The folic acid requirement for normal growth of poult on a purified ration is 2.0 mg per kilogram of feed.

At the 25% and 43% protein levels of the purified ration, 2.0 mg of folic acid per kilogram of feed is required to prevent cervical paralysis. No cervical paralysis occurred in the group which consumed the turkey starting mash.

When the level of folic acid was 2.0 mg per kilogram of feed, normal feathering was observed in the poult receiving the purified diet with 43% protein, but at the same folic acid level those fed the purified diet containing 25% protein showed feathering that was distinctly subnormal.

Although the hemoglobin values for the poult which did not develop cervical paralysis showed no direct relationship to the level of the folic acid supplement on either of the purified diets, they were higher, 11.6–12.7 gm, on the diet containing 43% protein than those on the diet containing 25% protein, 8.8–10.5 gm. Those obtained on the 43% protein diet were essentially of the same order as that observed with the poult on the turkey starting mash. For the birds which developed cervical paralysis, the hemoglobin values were lower and ranged from 5.7 to 7.3 gm per 100 ml of blood.

#### ADDENDUM

Since this manuscript was completed, Lillie and Briggs [Poultry Sci., 26: 295 (1947)] have reported that New Hamp-

shire chicks require a minimum of 150  $\mu$ g of folic acid per 2.0 mg of folic acid per kilogram of feed is required to prevent and that 200  $\mu$ g per 100 gm of diet (2.0 mg per kilo) is the requirement for normal feathering.

#### ACKNOWLEDGMENTS

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# THE NUTRITIVE VALUE OF CANNED FOODS<sup>1</sup>

## XXV. VITAMIN CONTENT OF CANNED FISH PRODUCTS

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Although a large number of canned meats and vegetable products have previously been assayed for vitamin content the number of fish products which have been thus investigated is relatively small. Recently it has been reported (Denel et al., '45) that at least some fish proteins are very complete with respect to the growth requirements of the rat. The following investigation was undertaken to determine the quantitative distribution of certain growth factors in different species of canned fish.

### EXPERIMENTAL

#### *Samples*

The method of collection of the samples has previously been outlined by Clifcorn ('44). Each sample consisted of 72 cans of the regular retail product and in most instances a report bearing a description of the location and date of catch, canning procedure, etc., accompanied each sample.

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The vitamin D assays reported in this paper were carried out at the Wisconsin Alumni Research Foundation laboratories under the direction of Dr. H. T. Scott, whose assistance is gratefully acknowledged. Thanks are due also to Mr. A. L. Powell, Jr., who did most of the thiamine determinations.

This work was a part of the National Canners Association-Can Manufacturer's Institute Nutrition Program.

Twelve cans were opened, the contents combined, and the solid and liquid portions separated with a fine screen. The liquid fractions were discarded except in the case of several of the products canned in vegetable oil. A few of these oils were analyzed for their vitamin A and D contents. The solids were then mixed by hand as thoroughly as possible, passed through a commercial meat grinder, mixed again and stored in the dark at a sub-zero temperature. All assays were carried out before this preparation was over 6 weeks old. For assays of vitamins A and D this material was used directly or if the oil was assayed it was simply clarified by filtration.

The water-soluble vitamin assays were carried out on a second preparation made from time to time by mixing an equal weight of the above material and distilled water in a Waring Blendor. All assays, except those for folic acid and vitamin D, were done in duplicate. The samples packed in tomato sauce were not separated into the solid and liquid fractions but the entire contents of the cans were prepared and assayed in the same manner as the solid portion referred to above.

The 11 samples of salmon assayed consisted of 5 different species of the genus *Oncorhynchus*, representing what are commonly called red, chinook, chum, coho and pink salmon.

### *Assay methods*

*Vitamin A.* Vitamin A was determined by the antimony trichloride method of Dann and Evelyn ('38) using the saponification procedure of Jensen and With ('39). It was found that the vitamin A content of the muscle tissue and oil was extremely low. In addition considerable carotenoid pigment appeared in the final chloroform extract although much of this could be removed by the separation procedure of Boyer, Phillips and Smith ('44). This separation procedure was used for the analyses of the oil from the Atlantic sardines and mackerel. The blue color of the vitamin A-antimony trichloride complex was much more stable in extracts made by



saponification of the oil than by saponification of the muscle of these 2 species.

Most species of salmon examined contained varying amounts of the astacin-like pigment described by Bailey ('37). In making saponifications of the whole muscle it was found that this pigment formed an alkali salt slowly and only after refluxing for a period of 2 hours was it almost completely removed with the saponified fraction. This technique apparently permitted fairly accurate checks to be obtained in the salmon analyses.

*Thiamine.* The thiochrome method of Hennessy ('42) as outlined in the U.S. Pharmacopocia ('47) was used for thiamine analyses. After incubation of the samples with the proteolytic and diastatic enzyme ("polidase")<sup>2</sup> the digests were extracted with ether to facilitate their passage through the adsorption columns.

*Riboflavin.* For riboflavin the microbiological method recommended by Strong ('47) was used. Since all samples contained appreciable quantities of fat the techniques suggested for its removal were employed.

*Pantothenic acid.* The *L. casei* method of Neal and Strong ('43) was used for pantothenic acid. The vitamin was liberated by digestion with "mylase P" according to Ives and Strong ('46). Precautions to remove fat were again exercised.

*Biotin.* *L. arabinosus* was used to measure biotin (Wright, '47).

*Nicotinic acid.* *L. arabinosus* was also used to assay nicotinic acid. The technique used was that of Snell ('47).

*Pyridoxine.* The *S. carlsbergensis* method of Atkin et al. ('43) was used with certain modifications of incubation procedure for the determination of pyridoxine activity. Either shaking in large test tubes (2.2 × 20 cm) or stagnant incubation in 125-ml Erlenmeyer flasks proved capable of providing the desired turbidity in 18-20 hours at 30°C.

*Folic acid.* In each case the samples for folic acid were treated with a crude conjugase preparation. The latter was

<sup>2</sup> Recommended by Clausen and Brown ('43).



prepared by disintegrating in a Waring Blendor, 1 part of fresh hog pancreas with 3 parts of water (weight to weight) and filtering to remove the large particles. This conjugase treatment usually increased the level of the vitamin from 50 to 70 fold as measured by the *L. casei* method of Teply and Elvehjem ('45).

TABLE 2  
*Vitamin D content of canned fish.*

SAMPLE <sup>1</sup>	USP VIT D UNITS PER 100 GM	SAMPLE <sup>1</sup>	USP VIT D UNITS PER 100 GM
Atlantic mackerel		Salmon	
1	250	1	500
2	250+	2	500
3	165	3	500+
4	250	4	500
Pacific sardines		5	500
1	333	6	500
2	333	7	500
3	333	8	500+
4	333	9	500
5	333	10	500
6	333	11	500
Atlantic sardines		Pacific sardines with tomato sauce	
1	250	1	333
2	333	2	333
3	250+	3	333
4	250	4	333
5	250	5	500
6	250+	Pacific mackerel	
7	333	1	250
8	250	2	250
Tuna		3	250
1	200	4	250
2	200	5	250
3	250	Atlantic sardine oil <sup>2</sup>	333
4	<100	Pacific sardine oil <sup>2</sup>	333
5	250	Tuna oil <sup>2</sup>	
6	225	3	333+
7	250	6	333
8	250	8	<100
9	250	12	333+
10	250		
11	250+		
12	333		

<sup>1</sup> Samples are the same as in table 1.

<sup>2</sup> Oils drained from the can contents.

*Vitamin D.* Rat assays for vitamin D were carried out according to the U.S.P. method. The amounts fed were such as to permit estimation of the potency with a precision indicated by the levels given in table 2, *e.g.*, 165, 200, 250, 333, 500 U.S.P. units per 100 gm. Closer estimation of the potency was not considered feasible on account of the excessive number of individual assay levels required.

#### DISCUSSION

Unfortunately very few analysts report the scientific name of the fish being sampled. This leads to some confusion as to whether, for example, a particular species is of Atlantic or Pacific origin and renders comparison with literature results rather difficult.

*Vitamin A.* Considerable variation was found in the vitamin A analyses of the different species of canned salmon. Thus 2 samples of red, 1 sample of sockeye (red) and 1 of chinook salmon contained 0.045, 0.045, 0.095 and 0.070 mg of vitamin A per 100 gm, respectively, whereas 3 samples of pink, 2 samples of coho and 2 of chum salmon ranged from 0.017 to 0.026 mg of vitamin A per 100 gm. No comparable variation in the B vitamin content of the salmon samples was observed.

Recently Awapara et al. ('46) have described a new spectrophotometric method for the determination of vitamin A. It is possible that this method could be applied to food samples where vitamin A is in small concentrations although it was unsatisfactory for measuring the vitamin in rat livers.

Since all samples contained some carotenoid pigment it was possible that higher values for vitamin A activity could be found by the rat assay. Devaney and Putney ('35) found 0.25 to 8.0 I.U. per gm of vitamin A in the flesh of several species of canned salmon. This range was approximately the same as reported in the present paper. The flesh of the other species assayed in the present work contained less than 0.008 mg of vitamin A per 100 gm. Until additional animal assay data are available it will

not be possible to draw final conclusions as to the value of canned fish as sources of vitamin A.

*Thiamine.* Guerrant, Vavich and Fardig ('45) have reported a survey carried out in a number of different laboratories. The thiamine content of a salmon purée varied between 0.0084 and 0.0104 mg per 100 gm when assayed by the thiochrome method. Some of the lower values reported in the present survey (table 1) may be due in part to thiaminase action in the muscle prior to canning. Thus Melnick et al. ('45) have found that herring contains this enzyme as does also California sardines (Lamb, '46). Myrback and Vallin ('44) have found that the canning process destroys 30% of the original thiamine in canned meat. These factors tend to decrease the value of canned fish products as a source of thiamine.

*Riboflavin.* The riboflavin content of the various samples within a given species showed remarkably little variation. In the Pacific sardines and mackerel the values were somewhat higher and can be regarded as comparable to lean beef or pork.

*Pantothenic acid.* All of the canned fish samples analyzed proved to contain approximately the same levels of pantothenic acid as those found in other muscle meats, namely, 0.25 to 0.83 mg per 100 gm for the fish as compared, for example, to 0.45 for pork (Guerrant, Vavich and Fardig, '45).

Very recently Lipmann et al. ('47) have reported that pantothenic acid may be the coenzyme for an acetylating enzyme for choline, sulfanilamide, etc. This discovery will add impetus to the search for more quantitative data on the distribution of this factor in foods.

However, the most serious problem in the estimation of pantothenic acid is its liberation from other materials in the sample. Incubation of the samples with "mylase P" was previously found to release more of the vitamin than treatment with other common proteolytic enzymes (Ives and Strong, '46); hence this technique has been applied in the present investigation. It is interesting to note that Lipmann

has found an enzyme in liver which together with a phosphatase liberated from the purified acetylating coenzyme 60 times as much pantothenic acid as could be obtained by clarase-papain digestion.

*Biotin.* Ives et al. ('46) found an average of 0.0098 mg of biotin per 100 gm in 10 samples of salmon assayed by the *L. casei* method of Shull and Peterson ('43). In the present study the Pacific sardines proved to be a somewhat better source of biotin than the other species.

There are several reports in the literature on the biotin activity of certain fatty materials such as, for example, oleic acid. It is possible that this factor may have been at least partially responsible for the fluctuations noted although all samples were filtered at a pH of 4.0-4.5 in order to remove fatty acids.

*Nicotinic acid.* All species can be stated to be a good source of nicotinic acid. The values found for the canned tuna samples were much higher than those usually reported in the literature for beef and ham.

*Pyridoxine.* The average pyridoxine value for the 10 salmon samples assayed by Ives et al. ('45) was 0.13 mg per 100 gm. The distribution of this vitamin in tuna and salmon, reported in table 1, is somewhat higher than most literature values for meat.

*Folic acid.* The assays reported for folic acid show that the various fish products are very low in this vitamin. The method of Roberts and Snell ('46) using *L. casei* as the test organism did not give more satisfactory results. There is some indication that this factor may be destroyed in the cooking of meat (Schweigert, Pollard and Elvehjem, '46), hence it is possible that the canning process may also destroy this factor.

Although the samples were treated with a conjugase preparation, it may be possible that not all the folic acid was actually liberated.

*Vitamin D.* The rather high levels of vitamin D found for the various species is one of the most interesting results of the present survey. From the values reported in table 2, it

may be seen that these species of fish are excellent sources of vitamin D and as such are unique among commonly eaten, unfortified foods. In view of the practical importance of this fact, the vitamin D values are given in full in table 2.

Devaney and Putney ('35) assayed 4 species of canned salmon and found the vitamin D content to range from 190 to 800 I.U. per 100 gm. The average figure taken from the results of the above authors is 460 I.U. per 100 gm, about the same value as that found in the present work (table 2).

It may be concluded that, with the exception of folic acid, the B vitamin content of the canned fish products assayed in the present study compares favorably with that of other major protein foods such as muscle meats, eggs, and cheese. In addition canned fish may be considered an excellent source of vitamin D, and should probably be regarded a rather poor source of vitamin A.

#### SUMMARY

A number of samples of canned fish representing 6 different species were tested for their content of vitamins A, D and certain water-soluble vitamins.

No appreciable amount of vitamin A was found in the flesh of any of the canned fish assayed except salmon, although small amounts were contained in the oils from canned Atlantic mackerel and sardines. Thiamine and folic acid were generally rather low. Riboflavin, pantothenic acid and biotin were present in fair amounts. The samples proved to be a good source of nicotinic acid and pyridoxine. All species examined were good sources of vitamin D.

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THE INFLUENCE OF PTEROYLGLUTAMIC ACID  
(A MEMBER OF THE VITAMIN M GROUP) ON  
GASTROINTESTINAL DEFECTS IN SPRUE.  
A STUDY OF INTERRELATIONSHIPS  
OF DIETARY ESSENTIALS<sup>1</sup>

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FIVE FIGURES

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Patients with the complete syndrome of sprue may exhibit the most remarkable combinations of deficiency states (Thay-  
sen, '32; Hanes, '46; Wintrobe, '46). Some of the mani-  
festations of these states are loss of weight (caloric defi-  
ciency), hypoproteinemia (protein deficiency), petechiae (vi-  
tamin K or C deficiency) (Fanconi, '38; Kark, Souter and  
Hayward, '40; Butt and Snell, '41), night blindness and  
xerosis conjunctivae (vitamin A deficiency) (Riddell, '33;  
Rao, '33), hypocalcemic tetany and osteoporosis (vitamin D  
and calcium deficiency) (Scott, '25; Linder and Harris, '30;  
Fairley, '36), and pancytopenia (vitamin M group deficiency)<sup>2</sup>

<sup>1</sup>This work was supported by grants from the National Vitamin Foundation, the Nutrition Foundation, Inc., the International Health Division of The Rockefeller Foundation, and Distillation Products, Inc.

<sup>2</sup>The term "deficiency" is employed here in its broad sense. We do not wish to imply that the deficiency is necessarily dietary. It is conceivable that some cases may be dietary in origin, but it is probable that some constitutional conditioning factor and/or environmental conditioning factor exerts a major influence upon the development of the disease-syndrome termed sprue.

(Darby and Jones, '45). In many cases these deficiencies may be classified (Dann and Darby, '45) as latent or potential and, hence, are demonstrable only by biochemical means.

The view that sprue is a primary disease was clearly argued by Thaysen ('32). Hanes ('43) held that the " . . . profound disturbance of the power of the intestines to absorb in a normal fashion . . . " would explain all of the varied secondary deficiency manifestations of the syndrome. The association of sprue with dietary inadequacies had often been remarked upon (Ashford, '22; Thaysen, '32), but Castle and associates ('35) clearly hypothesized that "In sprue the obvious dietary defects provide . . . a rational explanation of the subsequent defects of the specific functions of the alimentary tract demonstrated in patients." These gastrointestinal defects are at least partially corrected by a substance or substances present in liver extract (Castle et al., '35; Fairley, '36; Miller and Rhoads, '36; Barker and Rhoads, '37; Miller and Barker, '37).

With the demonstration of the activity of pteroylglutamic acid (PGA) in patients with sprue, we (Darby and Jones, '45; Darby, Jones and Johnson, '46a, b; Darby, Jones, Warden and Kaser, '47) indicated that this vitamin seemed to exert a favorable influence upon the gastrointestinal abnormalities as well as upon the anemia. Similar observations have been made by others (Beguerie and Spies, '46; Suárez, Spies and Suárez, '47). This paper summarizes additional data which may be interpreted to indicate that pteroylglutamic acid exerts a favorable influence upon the gastrointestinal tract in sprue.

#### EXPERIMENTAL

The subjects were 6 adult patients with sprue who have been observed in the Vanderbilt University Hospital. All of these cases met the diagnostic criteria of glossitis, diarrhea with an increased fat content of the stools, loss of weight, macrocytic anemia, moderate leucopenia and definite impairment of gastrointestinal absorption as will be discussed. Some of the initial data on 4 of these cases have been previously

reported (Darby, Jones and Johnson, '46a; Jones, Warden and Darby, '47). One patient (J.D.), however, has now been observed during a second relapse after the withdrawal of therapy and through a second remission induced by PGA. This makes a total of 7 observed remissions reported herein; each remission is considered independently. The sole specific therapeutic agent administered has been synthetic pteroylglutamic acid<sup>3</sup> in doses of either 5 mg per day orally or 15 mg per day intramuscularly, except in 1 case (Jones, Warden and Darby, '47) in which this was preceded by 10 days of therapy with pteroyltriglutamate. With 1 exception (J.H.), the patients have been hospitalized during the acute stage of their disease. The prehospital dietaries of the patients varied considerably. They had been generally of poor quality, but not uniformly so. During therapy 1 patient (P.B.) received a meat-free diet; the remainder have been maintained on a regular ward diet devoid of organ meats. No dietary advice whatsoever has been offered the patients upon their return home. Other than indicated in figure 2a no other supplementary vitamins have been administered.

### *Glossitis*

During each relapse the patient has complained of a sore tongue, particularly sensitive to acid foods, spices and the like. All of the patients have shown atrophic lingual papillae. Subjective relief of the glossitis has occurred and beginning regeneration of the papillae has been evident within 3 days to a week after the institution of therapy. In 6 of the 7 remissions induced by PGA there has been complete restoration to normal of the lingual changes. The other case (J.H.) has shown pronounced improvement. These successive lingual changes observed in J.D. prior to therapy, following administration of PGA, in a relapse after the interdiction of therapy,

<sup>3</sup> We are indebted to Drs. Stanton M. Hardy, Thomas H. Jukes, and E. L. R. Stokstad of the Lederle Laboratories for their generous assistance in supplying us with synthetic pteroylglutamic acid and the isolated pteroyltriglutamate employed in this study.

and in a second remission induced by PGA are shown in figure 1. If, as is sometimes held, the appearance of the tongue in patients with sprue is an indicator of the state of the gastrointestinal mucosa generally, these findings would

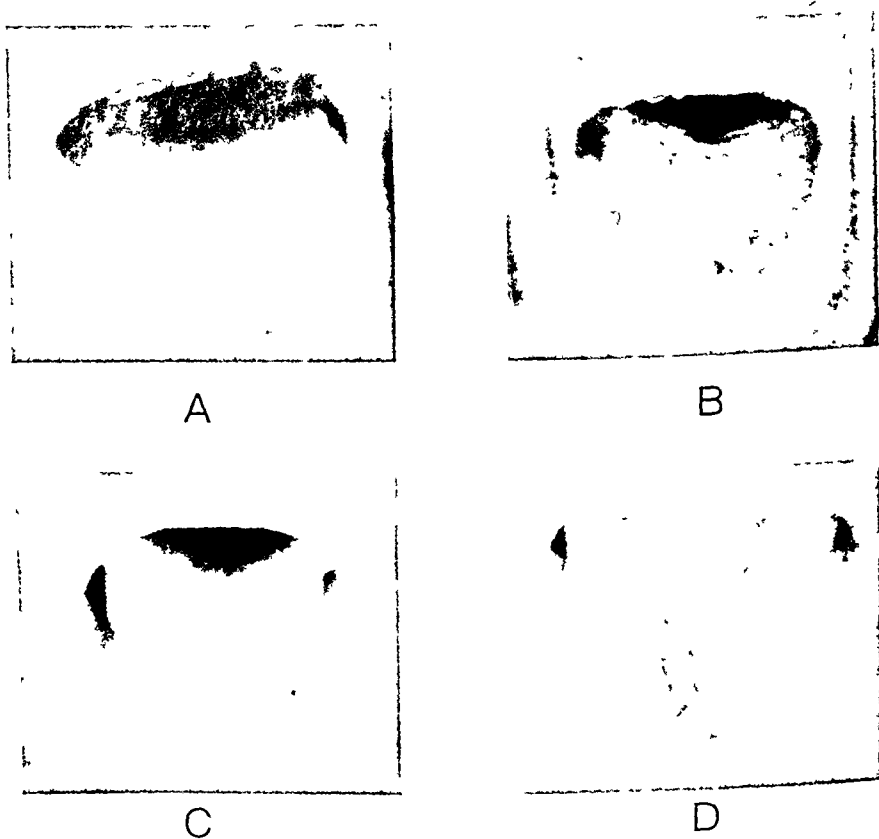


Fig. 1 a. Tongue of patient J.D. on September 26, 1945, prior to therapy. Note the complete absence of lingual papillae.

b. Tongue of patient J.D. photographed on October 18, 1945. Therapy with PGA was instituted on October 4, 1945. Note the evident regeneration of papillae which impart a velvety appearance to the central area.

c. Tongue of patient J.D. photographed on July 24, 1946, 275 days after the last injection of PGA. The patient was in a clinical relapse. Note the similarity in appearance to the photograph made on September 26, 1945.

d. Tongue of patient J.D. photographed on December 4, 1946, at the height of the second remission induced by PGA. Complete regeneration of the lingual papillae is evident.

indicate a definitely beneficial effect of this vitamin on the mucosa.

### *Diarrhea*

In all of these remissions cessation of diarrhea has been observed within a period of 3 to 9 days from the beginning of therapy with PGA. In 1 case (J.H.) periodic recurrence of this symptom has taken place, but the frequency of recurrence has decreased. As noted below, this patient has developed niacin deficiency while receiving PGA.

### *Weight changes*

The maximum weight gains in pounds have been as follows: 45, 26 (J.D.); 33 (P.S.); 24 (P.B.); 29 (W.O.); 6 (J.H.). It is our impression that these weight changes following therapy reflect both the increased appetite and the better absorptive capacity of the gastrointestinal tract with the consequent conservation of calories and fluid. Several of our patients have exhibited evidence of fluid retention during the early period of therapy (Darby, Jones and Johnson, '46a).

### *Glucose tolerance*

It has been possible to determine the glucose tolerance (oral administration) in 5 of these remissions. In each instance there has been a definite return toward the normal type of curve within from 11 to 30 days after institution of therapy. These changes are illustrated by figures 2a and 2b. Other data on the glucose tolerance were presented elsewhere (Darby, Jones and Johnson, '46a). The dosage of glucose employed in these studies has been 70 gm routinely. Confirmatory findings have been reported by Suárez et al. ('47). Crawford ('39) has interpreted the low glucose tolerance curves as indicative of defective or delayed absorption of carbohydrate from the intestine. Fairley ('36) regards the transition from the flat type of glucose tolerance curve to the normal type following therapy with liver extract as indicating improved absorption of carbohydrate. By similar

CORRELATIVE DATA ON PATIENT WITH SPRUE TREATED DURING TWO PERIODS WITH PGA

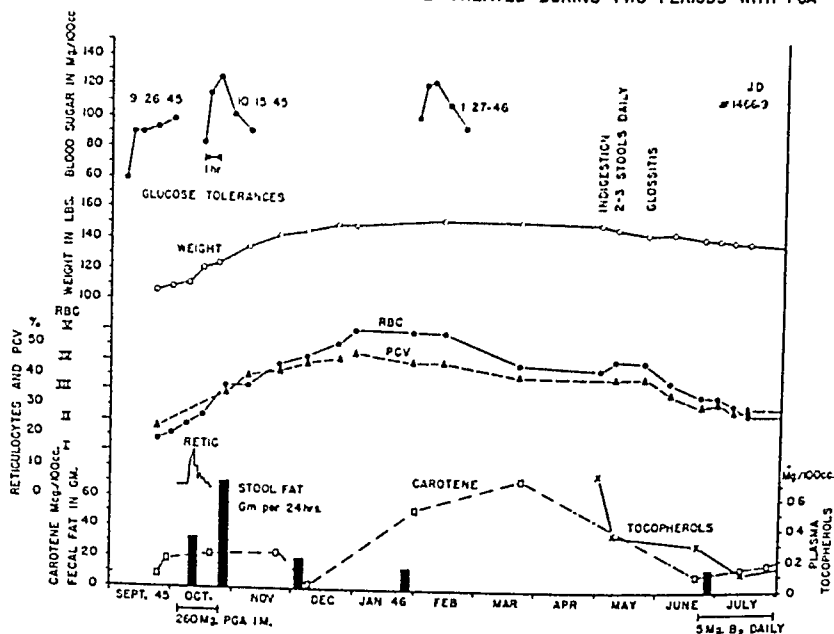


Fig. 2a See text for discussion. Riboflavin, 5 mg daily by mouth, was given as a placebo from June 26 to July 14, 1946. The course of the relapse was unaltered by this therapy.

reasoning, one may hypothesize that PGA favorably influences the permeability of the gastrointestinal tract to glucose.

### *Fat soluble factors*

It is essential that the dietary intake of fat be properly controlled (Black, Fourman and Trinder, '46) in order that quantitative analyses of stool fat be interpreted with accuracy. This control of the fat intake of patients is extremely difficult, especially after the subject returns home. Our observations on the effect of PGA on fecal fat are, therefore, exceedingly limited, but successive determinations on a single patient (figs. 2a and 2b) indicate a definite decrease in the total 24-hour loss of fat in the stool. In chronic sprue, however, PGA has not reduced the loss of fat in the stool beyond that point attained by previous liver extract therapy



(Davidson, Girdwood and Innes, '47). Spies et al. ('46) have observed that diarrhea was better controlled by PGA in patients whose illness was of recent onset.

CORRELATIVE DATA ON PATIENT WITH SPRUE TREATED  
DURING TWO PERIODS WITH PGA

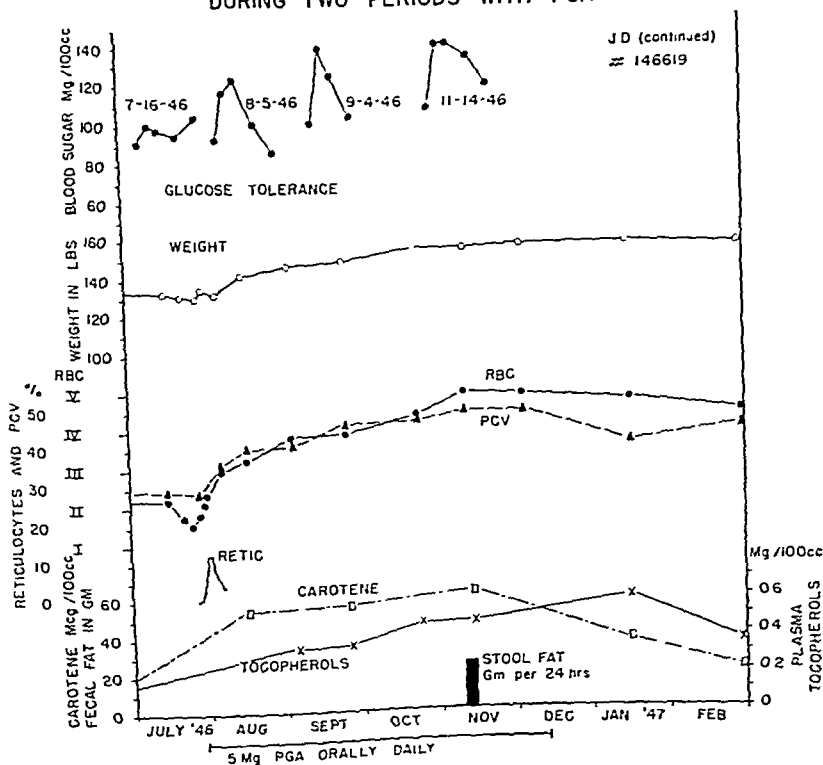


Fig. 2b See text for discussion.

### Carotene and vitamin A

Some data on the absorption of carotene and vitamin A have been presented in a previous communication (Darby, Kaser and Jones, '47). The hematologic pictures of sprue and pernicious anemia are identical and both diseases respond to PGA. It is of interest, therefore, to contrast the initial serum carotene levels of a group of patients with sprue with a group of patients with pernicious anemia (fig. 3). These

## CORRELATIVE DATA ON PATIENT WITH SPRUE TREATED DURING TWO PERIODS WITH PGA

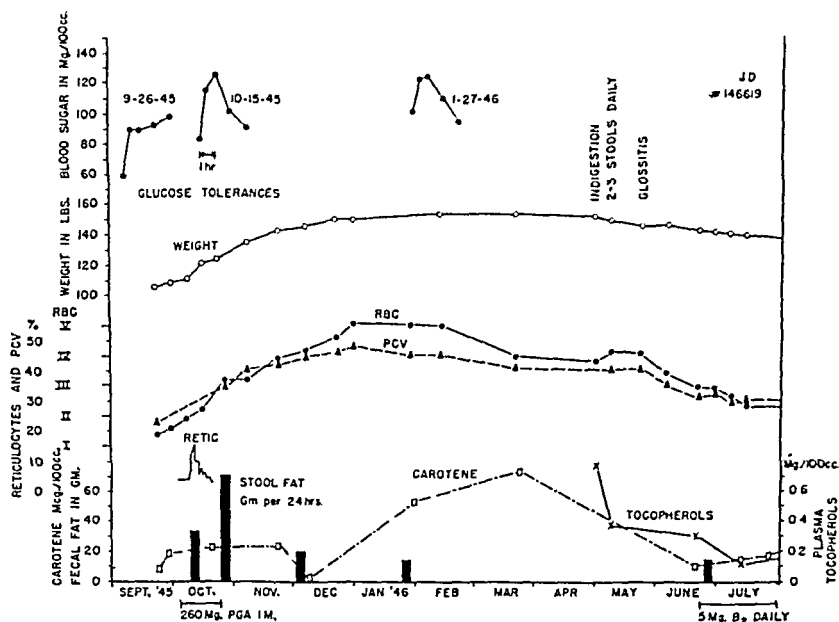


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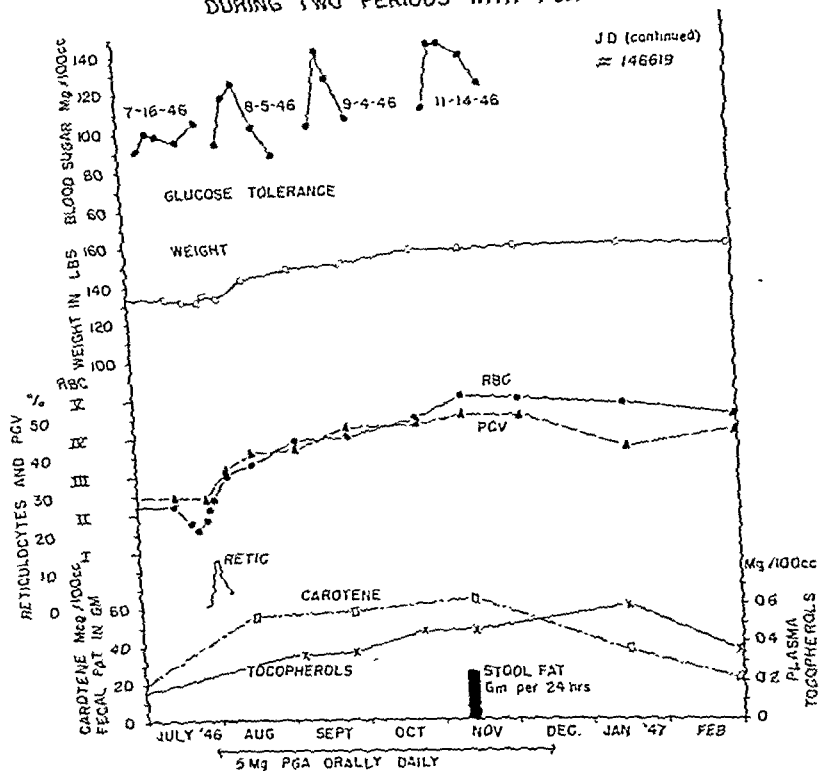


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findings are similar to those of Cayer and coworkers ('45, '46). Considered together with the normal-to-high glucose tolerance curves (Johnsson, '22; Hanes, '46) and the absence of the deficiency pattern on x-ray examination (Golden, '41) in pernicious anemia these findings indicate that the presence of the gastrointestinal defects is outstanding in sprue, but that this is not the case in pernicious anemia. The gastrointestinal abnormalities are not, then, secondary to macrocytic anemia.

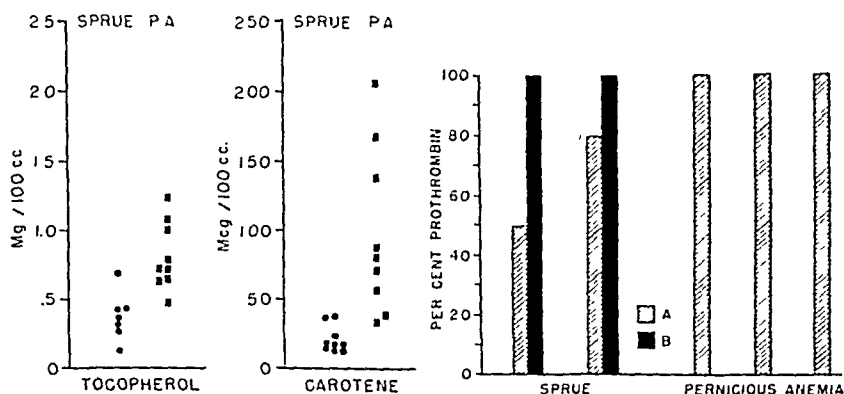


Figure 3

Fig. 3 Plasma tocopherol and serum carotene levels in sprue and pernicious anemia. The low initial carotene and tocopherol levels of patients with sprue are to be contrasted with the initial higher levels of patients with pernicious anemia in relapse. Carotene levels are included on 2 patients with sprue who were seen in other hospitals but are not reported in this paper.

Fig. 4 Plasma prothrombin levels in sprue and pernicious anemia. A, prothrombin concentration prior to therapy; B, prothrombin concentration after therapy with PGA. Note the contrast between the prothrombin concentrations in patients with sprue and pernicious anemia in relapse. The increase in prothrombin concentrations of the patients with sprue following therapy with PGA is not due to administered vitamin K.

The rise in serum carotene following therapy with PGA is illustrated in figure 2b and has been discussed in an earlier report (Darby et al., '47). This gradual rise may be attributed to the accumulation of carotene in the serum as the patient is better able to absorb the pigment. A sustained rise has been observed in 6 of the 7 remissions. The absence of a sustained carotene rise in 1 (J.H.) is partly explained

by the exceedingly low intake of carotene in his self-selected dietary (calculated to be 180 I.U. per day). In passing, the remarkable similarity of this increase in serum carotene content to the increased cholesterol levels in cases of sprue treated with liver extract (Fairley, '30) is to be noted.

Vitamin A absorption determinations following an oral dose of 200,000 I.U. of vitamin A have yielded data consistent with the interpretation that there was improved absorption in 2 of the 4 remissions in which repeated estimations were made. Two patients have continued to exhibit flat vitamin A tolerance curves despite clinical improvement. These individuals are persons with chronic sprue. One (P.B.) is an alcoholic; the other (J.H.) developed evidence of niacin deficiency following therapy with PGA.

### *Vitamin K*

The hypoprothrombinemia which can be observed in sprue is attributed to a deficiency of vitamin K (Kark, Souter and Hayward, '40). Two of our patients have exhibited hypoprothrombinemia prior to therapy with PGA and this hypoprothrombinemia has returned to normal during remissions induced by PGA (fig. 4). By way of contrast, the prothrombin times determined on 3 patients with pernicious anemia in relapse are included in this figure. Again the absence of a secondary fat-soluble vitamin deficiency in pernicious anemia is impressive.

### *Vitamin E*

Low plasma tocopherol levels have been reported in patients with sprue in relapse (Darby, Cherrington and Ruffin, '46). The decrease in serum tocopherol concentration during a relapse of sprue and the increase following therapy with pteroylglutamic acid are depicted in figures 2a and 2b. The contrast between the concentrations of fat-soluble vitamin E in the patients with sprue and in patients with pernicious anemia (fig. 3) is again noted.

Correlative data on many of the points discussed above are presented in figures 2a and 2b, which depict the course of patient J.D. during the 2 relapses and 2 remissions induced by PGA. These figures illustrate the rapidity with which alterations toward normal may occur in glucose tolerance following therapy and that this improved function may be maintained during a remission induced by PGA even after administration of the vitamin has been terminated. These figures also



Fig. 5 Gastrointestinal x-ray pattern as revealed 1 hour after a barium meal, patient J.H. The film on the left was obtained prior to institution of therapy, the one on the right 2 months after the beginning of treatment with 5 mg PGA daily. Note the less abnormal appearance of the intestines upon the second examination.

indicate a reciprocal relationship between the carotene level in the serum and the amount of fat lost in the stool. The very close time association between the hematologic and gastrointestinal changes observed during remission and relapse is consistent with the hypothesis that these are 2 manifestations of a deficiency<sup>4</sup> disease.

#### *X-ray changes in the gastrointestinal tract*

X-ray changes of the so-called deficiency pattern type are present in sprue (Mackie et al., '35; Golden, '41). Beguerie and Spies ('46) have reported that these return toward nor-

<sup>4</sup> See footnote 2 page 645.

mal following the administration of PGA. We have obtained follow-up films on patients in 4 remissions induced by PGA. In 3 of these definite improvement in the pattern has occurred following therapy with PGA (fig. 5). Less pronounced improvement has been observed in the fourth. In none of our cases have the x-ray patterns returned completely to normal despite the otherwise excellent response to treatment. Other types of specific therapy likewise fail to restore the intestinal pattern completely to normal in cases of sprue (Mackie, Miller and Rhoads, '35; Golden, '41).

#### DISCUSSION

Our observations may be interpreted as indicating that the primary defect in sprue is a deficiency state<sup>5</sup> manifested by glossitis, macrocytic anemia and pancytopenia, and impaired gastrointestinal function. This primary deficiency is corrected by pteroylglutamic acid. The simultaneous correction of several secondary deficiencies is also indicated by the data presented in this report. The numerous interrelationships of pteroylglutamic acid with other vitamins and food factors are a most remarkable clinical example of the interrelationship of foodstuffs in man. The close similarities to the multiple deficiency states (Daft and Sebrell, '45) in the sulfa-treated rat are obvious.

Our data lead us to suggest that PGA will probably produce effects similar to those of liver extract on the serum cholesterol (Fairley, '30), on serum calcium (Fairley, '36), on the sodium deficiency in sprue (Black, '46), and on other secondary deficiencies which occur in the disease.

Many cases of sprue undoubtedly develop irreversible changes in the gastrointestinal tract (Golden, '41; Hanes, '42). In 1 instance, Golden ('41) has demonstrated degenerative changes in the intrinsic nerve plexuses which supply the intestinal wall. McCarrison ('21) observed similar changes in monkeys which apparently had vitamin M deficiency (Day, '44). The vitamin M deficient monkey may be regarded as the

<sup>5</sup> See footnote 2 on page 645.

experimental analogue of sprue (Darby and Jones, '45; Darby et al., '46b; Jones et al., '47). It is improbable that completely degenerated nerve plexuses can be restored. Therefore, the persistence of some degree of malfunction despite therapy may be expected, particularly in disease of long standing. The failure of liver extract therapy to correct *in toto* the various disturbances of gastrointestinal absorption may be thus explained. If the above hypothesis holds, complete restoration of normal function of the gastrointestinal tract may never occur. The relationship of the anatomical findings to the functional defects in absorption is not clear. Further studies are planned in an effort to define the relative roles of the degenerative changes in the plexuses and the possible alterations in mucosal permeability in vitamin M deficiency.

It should be emphasized again that any disease which results in malabsorption from the gastrointestinal tract may resemble the sprue syndrome (Hanes, '46). Although the clinical pictures are indistinguishable, it is to be expected that PGA will be ineffective in the treatment of any disease which does not result from a deficiency<sup>a</sup> of this vitamin. Therefore, the availability of this chemically well-defined factor may enable clinicians to separate the different disease entities which are now grouped together loosely as the sprue syndrome or idiopathic steatorrhea. The reported inefficacy of PGA in certain cases of celiac disease (Davidson et al., '47) may indicate that this disease in children is on a different etiologic basis than is sprue in the adult. In this connection it is well to recall the similarity of the gastrointestinal x-ray pattern in celiac disease in children and sprue in adults to that of the very young normal infant (Golden, '41).

In the discussion of these findings we have adopted the convention of interpreting all of these data in terms of changes in gastrointestinal absorption. A critical appraisal of the evidence in the literature upon which this practice is based makes it seem likely that it is at least partially correct.

<sup>a</sup> See footnote 2 on page 645.



However, other interpretations are possible; for example, the observed changes in glucose tolerance and vitamin A tolerance could be explained on the basis of altered hepatic function or a combination of altered intestinal absorption and hepatic storage or release as has been observed for vitamin A in rats (McCoord et al., '47) and for vitamin A and carotene in the human with infective hepatitis (Harris and Moore, '47). Furthermore, it is well established that rats treated with sulfonamides manifest several vitamin deficiencies. The usual interpretation of these findings is that the sulfonamide inhibits the synthesis of the vitamins by the flora of the gastrointestinal tract. In the sulfa-treated rat there occurs a decrease in the coliform organisms in the gastrointestinal tract. These organisms are able to synthesize a number of vitamins (Daft and Sebrell, '45). In unpublished observations made in collaboration with Dr. John Buddingh, we have observed that the fecal flora of our patients with sprue is characterized by a decreased number of coliform organisms and an increased number of slow and non-lactose fermenting organisms. During therapy with PGA a normal flora seems to have been reestablished. It will require many careful studies over long periods of time to define the relative roles of altered gastrointestinal absorption, metabolism and gastrointestinal synthesis in the pathogenesis of the manifold deficiencies observed in sprue.

#### SUMMARY

Observations on 6 patients with sprue during 7 remissions following therapy with PGA have been summarized. Each remission has been characterized by relief of the glossitis, regeneration of the lingual papillae (fig. 1), cessation of the diarrhea, and gain in weight, as well as hematologic improvement (figs. 2a and 2b). In 5 instances a return toward the normal glucose tolerance following therapy was demonstrated. A gradual increase in the serum carotene concentration was observed in 6 of the remissions and improved vitamin A absorption and increased prothrombin concentration (fig. 4) demonstrated in 2 instances each. Increased concentrations of

plasma tocopherol have been observed in 2 cases, and a decreased loss of fat in the stool has occurred in the 1 patient who was studied repeatedly (figs. 2a and 2b).

These findings are compatible with an interpretation that PGA exerts a favorable influence on the primary gastrointestinal disorder in sprue and thereby permits the correction of many secondary deficiencies which occur. Other possible interpretations are indicated.

Chronic sprue responds less completely to PGA than does the more acute disease.

As further evidence for the effectiveness of PGA in sprue, 2 patients have relapsed when therapy with PGA was withheld. All of the manifestations of the disease reappeared (figs. 2a and 2b).

Sprue and pernicious anemia differ in that gastrointestinal defects are pronounced in sprue.

#### ACKNOWLEDGMENTS

We wish to express our sincere appreciation to the members of the Medical Staff of Vanderbilt University Hospital who have assisted us in the handling of these patients, to Dr. Ann S. Minot for assistance and advice on several of the chemical determinations, to Dr. H. C. Francis for his interpretation of the roentgenologic findings, and to the Staff of the Tennessee Vanderbilt Nutrition Project for technical assistance.

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# MINERAL METABOLISM STUDIES IN DAIRY CATTLE

## I. THE EFFECT OF MANGANESE AND OTHER TRACE ELEMENTS ON THE METABOLISM OF CALCIUM AND PHOSPHORUS DURING EARLY LACTATION<sup>1</sup>

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The increasing capacity of the dairy cow for milk production has suggested to many investigators the possible need of supplying mineral elements to the cow in quantities greater than that provided by home-grown feeds. Numerous studies have resulted in the drawing of variable conclusions in regard to an adequate calcium and phosphorus supply for lactating cows. Generally the requirements are thought to be approximately 10 gm each daily, of calcium and phosphorus for a 1000-pound milking cow and an additional allowance of 0.9 gm calcium and 0.7 gm phosphorus per pound of milk produced as proposed by Huffman ('34).

Numerous balance trials have demonstrated the occurrence of negative calcium and, somewhat less frequently and for shorter periods of time, negative phosphorus balances during the early portion or peak of lactation. Ellenberger and associates ('31 and '32) and Huffman et al. ('30a) studying cal-

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cium and phosphorus metabolism during the gestation, lactation and dry periods found negative balances during peak production followed by a replenishing of the stores of these elements during the drying-off and dry periods. This has been considered to be a normal cycle during which the importance of the dry period was emphasized (Bohstedt, '42 and Hart et al., '31).

A series of extensive investigations made by Hart and associates to determine some of the dietary factors influencing the assimilation of calcium in cows and/or goats dealt with calcium ('23 and '27a), phosphorus ('23), cod liver oil ('27b and '29a) and irradiated yeast ('30) supplementation; the feeding of green grasses ('23a, '27a and '31), sun-cured ('29b, and Steenbock et al., '25) and weathered hays ('25); the feeding of glucose ('31) and hydrochloric acid ('31) and the exposure of animals to direct sunlight ('26 and '27a) and other ultraviolet light radiations ('24, '26 and '27c). These workers concluded from their experiments that there are factors other than vitamin D operating in the absorption of calcium from the intestinal tract of the cow.

There are no reports found in the literature which would indicate that manganese, iodine, copper, cobalt, iron or zinc influences the assimilation of calcium in lactating cows. Palmer et al. ('28), however, demonstrated that a high magnesium ration decreased the assimilation of calcium and phosphorus in cows. The effect was most marked when the phosphorus content of the ration was low. More recently, Huffman and co-workers ('30b) showed that the inclusion of 3 to 5% of magnesium salts in the rations of calves maintained on a low calcium ration either had no effect at all or slightly improved the retention of calcium and phosphorus. These investigators (Huffman and Duncan, '35) later demonstrated that magnesium improves calcium and phosphorus metabolism and bone calcification in dairy calves and described this function as a vitamin D-sparing effect.

The ingestion of beryllium salts was shown to lead to a type of rickets caused by the formation of insoluble beryllium phos-

phate in the intestinal tract (Kay and Byrom, '27). Apparently other metallic salts such as aluminum, thallium, barium, strontium and magnesium act similarly (Schmidt and Greenberg, '35).

Perosis, a disease indicated by slipped tendons in chickens, was proven by Wilgus and associates ('36) to be caused by a deficiency of manganese. Other investigations, dealing with the role of manganese in calcium and phosphorus metabolism, demonstrated a favorable influence of this element on the development of bone in rats (Amdur et al., '45, and Wachtel et al., '43), pigs (Miller et al., '40) and rabbits (Smith et al., '44).

In a study conducted by Chornock et al. ('42) using rats, an increased calcium and phosphorus excretion resulting in negative balances was effected by increasing the manganese intakes.

Reports in the literature on the effects of various mineral elements upon the metabolism of calcium and phosphorus in dairy cattle are limited except in the case of magnesium.

This experiment was initiated to study the effects of supplemental calcium and manganese and other mineral element supplementation upon the metabolism of calcium and phosphorus in dairy cows during the early portion of lactation.

#### EXPERIMENTAL

During this investigation the metabolism of calcium and phosphorus was studied by the balance trial method. Three weekly balance trials were conducted at regular intervals with each of 8 Holstein and 4 Guernsey cows during the first 5 months of lactation.

The 12 cows comprised 3 collection and 4 feed groups. The collection groups, composed of 4 animals from which excreta was collected while on trial simultaneously, were selected as nearly uniformly as possible relative to size, age, breed and stage of lactation. The feed groups received the following grain mixtures continuously during the 5-month feeding trial: group I, basal grain mixture (consisting of

ground corn, 30 parts; rolled oats, 30 parts; linseed meal, 28 parts; wheat bran, 11 parts and plain salt, 1 part); group II, basal grain mixture plus the addition of 3 pounds of  $\text{CaCO}_3$  (c.p.) per 100 pounds of the basal grain ration, group III, basal grain mixture plus the addition of 3 pounds of a mixture of  $\text{CaCO}_3$  (c.p.) and  $\text{MnSO}_4$  (c.p.) (proportions of calcium and manganese same as in Mico<sup>3</sup>) per 100 pounds of basal grain mixture; group IV, basal grain mixture plus the addition of 3 pounds of Mico<sup>3</sup> per 100 pounds of basal grain mixture. Vitamin D supplements were not given to any of the groups.

The general feeding plan provided 1 pound of timothy-clover hay with 3.5 pounds of corn silage per 100 pounds of body-weight daily and grain in sufficient quantity to satisfy the Morrison feeding standards ('39) plus 10%.

The feces and urine were collected separately by attendants. Weekly composites of daily aliquots of silage, hay, water, milk, feces and urine were analyzed for calcium and phosphorus according to the official methods of the A.O.A.C. ('40).

The chemical methods used to determine the levels of certain blood and plasma constituents at intervals during the feeding trial are as follows: hemoglobin, Sanford, Sheard and Osterberg ('33); total plasma proteins, Looney and Walsh ('39); calcium, Clark and Collip ('25) and inorganic phosphorus, Fiske and Subbarow ('25). The red blood cell count and volume (hematocrit) were determined on the same blood samples according to the standard procedures.

The "percentage used" of calcium and of phosphorus was determined by dividing the algebraical sum of the balance and the quantity of the element in the milk by the quantity of the element taken in by the animal.

## RESULTS AND DISCUSSION

### *Health and milk production*

No differences were found in the general health of the animals of the 4 feed groups. It is not known, however, what

<sup>3</sup>Mico has the following percentage composition: calcium 33; magnesium 2; manganese 0.20; iron 0.20; iodine 0.045; copper 0.025; zinc 0.01 and cobalt 0.002.



would be the effects of the different supplements used or the lack of them over an entire lactation period or over the life-time of an animal.

During the relatively short period of experimentation, no definite effects on the production of the cows were found as a result of calcium supplementation alone or of calcium plus trace elements supplementation; the control animals (group I) seemed to maintain their levels of production equally as well as the groups receiving the various supplements. The high milk production of group I as compared to the other groups is largely the reflection of the high production of cow 391 which possessed an inherently greater capacity for production than the other animals.

### *Blood picture*

Although the hemoglobin levels, red blood cell counts and volumes of the blood of the animals of the 4 groups were not greatly different, these values were somewhat higher for groups II, III and IV than for group I (table 1).

Table 1 also shows no apparent group difference in the average levels of plasma calcium and phosphorus or in the levels of plasma proteins.

### *Calcium metabolism*

Tables 2-5 show the average daily milk production, number of days in milk, and the intake, outgo, balance and percentages of calcium and phosphorus used for all animals of groups I, II, III and IV, respectively. It will be noted that each animal appeared on trial 3 times and that the metabolism period date indicates the sequence in which the trials were conducted. A general summary of group average calcium and phosphorus metabolism data and individual animal histories are given in tables 6 and 7, respectively.

Tables 2-5 show that during the 9 trials run on each group the following number of negative calcium balances were encountered in decreasing order of occurrence: group III, 9;

TABLE 1  
Average blood picture of cows on different rations during feeding period (mean  $\pm$  standard deviation).

GROUP	PLASMA		WHOLE BLOOD			
	Ca	Inorg. P	Hemoglobin		R.B.C.O.	R.B.O.V.
			Total proteins	gm %		
I	10.61 ± 1.09	5.41 ± 0.77	8.30 ± 0.75	9.97 ± 1.37	6.24 ± 0.85	27.85 ± 4.04
II	10.45 ± 0.86	5.12 ± 0.85	7.75 ± 0.36	10.70 ± 1.58	6.95 ± 0.63	31.14 ± 2.17
III	10.70 ± 0.83	5.63 ± 0.75	7.83 ± 0.39	10.70 ± 1.07	7.51 ± 1.11	29.84 ± 3.70
IV	10.47 ± 0.78	5.18 ± 0.89	7.90 ± 0.55	11.14 ± 1.63	7.65 ± 0.73	31.87 ± 2.81

TABLE 2

*Average daily calcium and phosphorus metabolism of cows fed a basic ration of timothy-clover hay, corn silage and grain.*

ANIMAL	METABOLISM PERIOD DATE	DAYS IN MILK WHEN ON TRIAL	AVERAGE DAILY MILK	RATIO CA/P IN FEED	CALCIUM				PHOSPHORUS			
					Intake	Output	Balance	Used <sup>1</sup>	Intake	Output	Balance	Used <sup>1</sup>
391	11/24-11/30/45	56-63	25.36	0.93	gm	gm	gm	%	gm	gm	gm	%
566	12/4-12/10/45	29-36	17.68	1.20	54.14	60.14	-11.08	27.68	66.27	66.97	-0.70	27.81
610	12/13-12/19/45	45-52	17.77	0.92	54.14	60.14	-6.00	23.72	45.00	47.44	-2.44	33.87
391	1/6-1/12/46	100-107	23.85	0.83	42.00	50.61	-8.61	42.05	45.76	47.02	-1.26	39.70
566	1/16-1/22/46	73-80	18.37	0.96	53.11	62.98	-3.45	35.95	72.06	73.12	-1.06	25.21
610	1/25-1/31/46	88-95	14.42	0.61	39.11	49.37	-3.74	42.41	52.30	57.47	-2.17	36.32
391	2/14-2/20/46	139-146	20.91	0.65	51.90	40.96	-1.85	42.63	64.35	63.38	0.97	25.34
566	2/23-3/1/46	112-119	17.59	0.60	38.11	54.31	-2.41	38.48	79.13	69.13	10.00	36.03
610	3/6-3/12/46	121-128	16.39	0.54	32.12	45.35	-7.24	27.75	63.28	61.63	1.65	33.50
					32.12	36.40	-4.28	54.84	59.08	61.68	-2.60	29.07

<sup>1</sup>The "percentage used" of calcium and phosphorus was calculated by dividing the algebraical sum of the balance and the quantity of the element in the milk by the quantity of the element ingested by the animal. In this derivation milk is considered as a body constituent.

TABLE 4

Average daily calcium and phosphorus metabolism of cows fed a basic ration of timothy-clover hay, corn silage and grain supplemented with calcium carbonate and manganese sulfate.

ANIMAL	METABOLISM PERIOD DATE	DAYS IN MILK WHEN ON TRIAL	AVERAGE DAILY MILK kg	RATIO CA/P IN FEED	CALCIUM			PHOSPHORUS				
					Intake gm	Output gm	Balance gm	Intake gm	Output gm	Balance gm		
529	11/24-11/30/45	56-63	13.80	2.34	92.48	95.27	-2.79	10.55	39.45	42.47	-3.01	16.97
552	12/4-12/10/45	49-56	14.66	2.41	90.01	93.00	-2.99	13.09	37.39	33.39	3.52	39.75
765	12/13-12/19/45	40-47	18.07	2.27	117.56	122.66	-5.10	15.31	51.82	50.20	1.62	37.93
529	1/6-1/12/46	100-107	10.93	2.13	88.57	81.37	7.20	18.48	41.65	41.83	-0.18	20.87
552	1/16-1/22/46	93-100	13.82	2.25	95.95	78.02	17.93	33.34	42.60	44.33	-1.73	25.37
765	1/25-1/31/46	83-90	16.52	1.98	117.60	114.93	2.67	19.09	58.49	58.17	0.32	29.70
529	2/14-2/20/46	139-146	7.30	1.92	85.60	86.02	-0.42	6.66	44.49	40.50	3.99	23.68
552	2/23-3/1/46	132-139	13.84	1.81	87.78	88.29	-0.51	15.74	48.59	45.57	3.02	30.53
765	3/6-3/12/46	116-123	19.55	1.87	117.53	110.04	7.49	26.62	62.87	64.40	-1.53	33.48

ANIMAL	METABOLISM PERIOD DATE	DAYS IN MILK WHEN ON TRIAL	AVERAGE DAILY MILK kg	MAN-URINE ADDED TO RATION	RATIO CA/P IN FEED	CALCIUM			PHOSPHORUS				
						Intake gm	Output gm	Balance gm	Intake gm	Output gm	Balance gm		
530	11/24-11/30/45	50-57	18.23	0.41	2.22	119.95	123.99	-4.04	10.71	54.12	52.67	1.45	29.13
569	12/4-12/10/45	34-41	19.22	0.18	2.34	75.27	85.99	-10.72	3.65	32.13	33.71	-1.57	32.37
733	12/13-12/19/45	57-64	16.03	0.40	2.18	105.52	117.99	-12.47	7.20	48.45	46.89	1.56	39.15
530	1/6-1/12/46	94-101	16.34	0.41	2.04	115.47	120.56	-5.09	8.55	56.53	54.43	2.10	27.89
569	1/16-1/22/46	78-85	11.77	0.25	2.17	80.90	84.53	-3.63	10.99	37.22	38.88	-1.66	26.30
733	1/25-1/31/46	100-107	14.35	0.44	1.87	103.62	115.62	-11.98	5.45	55.26	57.18	-1.92	24.85
530	2/14-2/20/46	133-140	14.46	0.41	1.69	106.49	110.64	-4.15	9.29	62.72	54.11	8.61	33.90
569	2/23-3/1/46	117-124	10.78	0.25	1.69	60.31	80.40	-14.09	-3.72	41.65	39.07	2.58	32.05
733	3/6-3/12/46	133-140	13.91	0.47	1.61	101.03	110.26	-9.23	8.23	62.84	60.49	2.35	28.77

Average daily calcium and phosphorus metabolism of cows fed a basic ration of timothy-clover hay, corn silage and grain supplemented with calcium carbonate and manganese sulfate.											
TABLE 4											

TABLE 5

*Average daily calcium and phosphorus metabolism of cows fed a basic ration of timothy-clover hay, corn silage and grain supplemented with Mico.*

ANIMAL	METABOLISM PERIOD DATE	DAYS IN MILK WHEN ON TRIAL	AVERAGE DAILY MILK	MAN- GANESE ADDED TO RATION	RATIO CA/P IN FEED	CALCIUM			PHOSPHORUS		
						Intake	Outgo	Balance	Intake	Outgo	Balance
			kg	gm		gm	gm	gm	gm	gm	gm
386	11/24-11/30/45	72-79	16.80	0.31	2.25	113.13	109.72	3.42	18.05	50.17	48.14
557	12/4-12/10/45	50-57	16.23	0.30	2.23	103.91	106.30	-2.39	15.65	46.67	42.36
718	12/13-12/19/45	46-53	14.84	0.33	2.07	84.46	71.54	12.92	35.96	40.81	37.07
386	1/6-1/12/46	116-123	14.90	0.31	2.13	115.30	106.06	9.27	20.11	54.17	49.60
557	1/16-1/22/46	94-101	13.86	0.30	2.21	103.87	87.83	16.04	29.67	46.90	49.13
718	1/25-1/31/46	89-96	13.46	0.45	1.88	103.33	90.08	13.25	28.46	54.82	54.64
386	2/14-2/20/46	155-162	12.57	0.31	1.82	105.15	104.88	0.27	12.43	57.79	52.88
557	2/23-3/1/46	133-140	12.21	0.27	1.63	77.94	81.58	-3.64	12.65	47.74	46.28
718	3/6-3/12/46	122-129	12.06	0.41	1.65	90.91	78.56	12.35	30.05	55.23	53.77

TABLE 6

*Group average daily milk production, days in milk and calcium and phosphorus intakes, balances and percentages used (mean  $\pm$  standard deviation).*

GROUP	AVERAGE DAILY MILK	DAYS IN MILK	MANGANESE ADDED TO RATION	CALCIUM			PHOSPHORUS		
				Intake	Balance	Used <sup>1</sup>	Intake	Balance	Used <sup>1</sup>
I	19.14 $\pm$ 3.55	88.3 $\pm$ 37.0		47.97 $\pm$ 10.40	-4.58 $\pm$ 4.34	37.35 $\pm$ 9.76	61.14 $\pm$ 10.98	0.27 $\pm$ 3.93	31.76 $\pm$ 5.28
II	14.28 $\pm$ 3.67	93.3 $\pm$ 36.0		99.23 $\pm$ 14.05	2.61 $\pm$ 7.23	17.65 $\pm$ 8.14	47.48 $\pm$ 8.74	0.67 $\pm$ 2.51	28.70 $\pm$ 7.66
III	14.23 $\pm$ 2.39	91.9 $\pm$ 36.2	0.36 $\pm$ 0.10	97.17 $\pm$ 18.58	-8.38 $\pm$ 4.16	6.71 $\pm$ 4.57	50.10 $\pm$ 11.01	1.50 $\pm$ 3.24	30.47 $\pm$ 4.41
IV	14.10 $\pm$ 1.72	100.9 $\pm$ 37.3	0.33 $\pm$ 0.06	99.78 $\pm$ 12.66	6.83 $\pm$ 6.61	22.56 $\pm$ 8.63	50.48 $\pm$ 5.44	2.27 $\pm$ 2.36	30.07 $\pm$ 6.37

<sup>1</sup> The "percentage used" of calcium and phosphorus was calculated by dividing the algebraical sum of the balance and the quantity of the element in the milk by the quantity of the element ingested by the animal. In this derivation milk is considered as a body constituent.

group I, 8; group II, 5 and group IV, 2. Since the stage of lactation was similar for the animals of each group, table 6 embodying average figures (representing 9 balance trials) is presented by groups for comparison purposes. These data show that the percentages of calcium used by the 4 groups in decreasing order were: group I,  $37.35 \pm 9.76$ ; group IV,  $22.56 \pm 8.63$ ; group II,  $17.65 \pm 8.14$  and group III,  $6.71 \pm 4.57$ . The control group cows receiving a low calcium ration utilized more of this element than the other groups, which substantiates the early work on this problem (Huffman et al., '30a).

It will be noted in table 6 that group III receiving the basal ration supplemented with  $\text{CaCO}_3$  and  $\text{MnSO}_4$  and milking an average of  $91.9 \pm 36.2$  showed an average daily calcium balance of  $-8.38 \pm 4.16$  gm and  $6.71 \pm 4.57\%$  used as compared to  $2.61 \pm 7.23$  gm and  $17.65 \pm 8.14\%$ , respectively, for group II receiving the basal ration supplemented with  $\text{CaCO}_3$  and milking an average of  $93.3 \pm 36.0$  days. (When the data for animal 552 during metabolism period 1/16-22/45, which are not of comparable magnitude with the other data of group II, are eliminated, the balance and percentage of calcium used by this group are  $0.69 \pm 4.69$  and  $15.69 \pm 6.02\%$ , respectively). The average daily calcium intake for the cows of groups III and II was  $97.17 \pm 18.58$  and  $99.23 \pm 14.05$  gm, respectively. Since the average daily milk production was  $14.23 \pm 2.39$  kg and  $14.28 \pm 3.67$  kg for groups III and II, respectively, the theoretical demand for calcium was similar for both groups, other than the requirements for maintenance (cow 569 was the only pregnant cow in groups II or III and was carrying a month-old fetus during her last trial period. The apparent depression of the metabolism of calcium in the animals of group III was attributed to the  $\text{MnSO}_4$  ingested. It seems unlikely that the greatly reduced utilization of calcium can be explained by reference to the phosphatase enzyme system, as the primary effects of any acceleration of phosphatase activity by manganese as reported by Wiese et al. ('38) should be reflected in the metabolism of phosphorus. Although group III was slightly higher than group II

Salter ('40) stated that an increased thyrotropic activity of the pituitary was found during pregnancy and lactation. This may be associated with the negative calcium balances reported for cows during early lactation. Goormaghtigh and Handovsky ('35) and Nitschke ('33) attributed a thyrotropic action to vitamin D when administered in small doses and a depression of thyroid activity when large doses were given; this effect was thought to concern the metabolism of iodine. Campbell ('42) offered data supporting the belief that vitamin D tends to spare the parathyroid gland.

Although literature concerning the relationship between the sex glands, the anterior pituitary and mineral metabolism in the bovine is limited, Riddle and Dotti ('45) showed that the follicular hormone increases the serum calcium concentration of castrated animals of other species. These workers also showed that gonadotropins increase the plasma calcium level only in birds whose ovaries are sufficiently matured to produce estrogen, but are ineffective in males and ovariectomized fowl. This study did not determine whether the follicular or the luteinizing gonadotropin is chiefly concerned. Riddle and Dotti ('45) further demonstrated that estrogenic hormones (estrone, estradiol, estriol and diethylstilbestrol) have a specific ability to increase the bound calcium fraction in the plasma of doves, pigeons, and other fowl of both sexes. The latter investigation showed that these hormones are equally effective in normal, hypophysectomized, castrated, thyroidectomized and adrenalectomized pigeons. The androgens, however, did not affect the plasma calcium whatsoever. Progesterone showed only slight ability to increase the plasma calcium level.

The literature seems to indicate that various sex and other hormones may have a greater role in calcium metabolism, especially during early lactation, than was previously supposed, and that some of the mineral elements may indirectly affect the metabolism of calcium through some of the enzyme or endocrine systems.

*Phosphorus metabolism*

The data shown in tables 2-5 for the metabolism of phosphorus indicate that the balance and utilization of phosphorus were similar for all groups regardless of the supplement fed. However, groups III and IV which received supplemental manganese in addition to calcium showed slightly higher phosphorus balances than group I or II not receiving supplementary manganese, although only negligible differences existed in the percentages of phosphorus utilized. The number of negative phosphorus balances in order of increasing frequency were: group IV, 1; group III, 3; group II, 4 and group I, 6.

## SUMMARY

A study was made of the effects of calcium and manganese and other mineral element supplementation upon the metabolism of calcium and phosphorus during the first 5 months of lactation of 8 Holstein and 4 Guernsey cows.

Calcium equilibrium was maintained most frequently in the group of cows receiving Mico<sup>3</sup> as the supplement to a basal ration of grain, corn silage and timothy-clover hay, and seemed to be attributable to the additional intakes of iodine, magnesium, copper, cobalt, zinc and iron supplied by this product.

Supplementation of the same basal ration with  $MnSO_4$  in addition to  $CaCO_3$  resulted in negative calcium balances in every case, whereas several positive balances occurred and higher percentages of calcium were used when the basal ration was supplemented with  $CaCO_3$  alone. The marked depression of calcium metabolism appeared to be effected by  $MnSO_4$  supplementation.

Phosphorus metabolism was not appreciably affected by the supplements used in this experiment.

<sup>3</sup> See footnote 3 on p. 664.

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# THE EFFECTS OF CALORIC RESTRICTION ON SKELETAL GROWTH

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FOUR FIGURES

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It has long been recognized that the ingestion of inadequate quantities of a complete diet (chronic partial inanition) results in a cessation of bone growth. Recently, it was observed that the bones of young rats, on diets containing large quantities of lactose or galactose, failed to grow in length or width (Handler, '47). These animals ingested adequate quantities of a high calcium and phosphorus diet and, in fact, absorbed unusually large amounts of calcium from the intestinal contents because of the presence of the lactose (or galactose). However, these rats also failed to increase in weight because of a defective carbohydrate metabolism, suggesting that growth and calcification of the skeleton, like growth of soft tissues, are also dependent upon an adequate caloric supply and metabolism. The present paper describes the effects of simple caloric restriction with an otherwise adequate dietary regimen, on skeletal calcification and growth.

## EXPERIMENTAL

The animals used were male rats of the Vanderbilt strain (Wolfe, Bryan and Wright, '38). The composition of the diets employed is given in table 1. It was arbitrarily decided to adopt, as the maximal limit of caloric restriction, approxi-

mately 50% of the *ad libitum* intake of normal controls of the same age and weight. By doubling the concentration of all dietary components save the sucrose of diet A, and then permitting the animals on diet B to eat only half as much as those on diet A, an intake of all essential nutrients (vitamins, minerals, proteins) equal to those of the *ad libitum* controls on diet A was assured. This procedure resulted in a caloric restriction of about 50% since diets A and B were virtually equicaloric. Diet C, when restricted to an intake equal to 50% of that of the controls on diet A, also resulted in a 50%

TABLE 1  
*Percentage composition of diets.*

	A	B	C	D
Casein	25	50	25	40
Sucrose	67	34	59	48
Cod liver oil	4	8	8	6
Salt mixture <sup>1</sup>	4	8	8	6

In addition, each kilo of diet A contained thiamine chloride 4 mg, riboflavin 5 mg, pyridoxine 4 mg, calcium pantothenate 30 mg, nicotinic acid 10 mg,  $\alpha$ -tocopherol 20 mg, naphthohydroquinone acetate 5 mg, *p*-aminobenzoic acid 50 mg, inositol 100 mg, choline chloride 250 mg. Each of these was doubled in diets B and C and increased 50% in diet D.

<sup>1</sup> Hubbell et al. ('37).

caloric restriction but the protein intake was also 50% of that of the controls. Diet D was so arranged that rats ingesting 75% as much food as *ad libitum* controls on diet A would receive identical supplies of all essential nutrients but only 75% of the caloric intake. The adequacy of intestinal absorption of diets B, C and D under such circumstances remains an uncontrolled problem but there is no definite reason to suspect that it was faulty. The salt mixture used in these studies was one which provides rather more calcium and phosphorus than most salt mixtures used for such studies (Hubbell, Wakeman and Mendel, '37) and contains 21.5% calcium and 5% phosphorus. The dietary supply of these minerals, like that of the other essential nutrients was therefore adequately insured.

Such studies were made on a large number of rats but the data presented in table 2 are given only for 1 such experiment. The results, however, are quite typical of all the series. Rats of 2 ages were studied. Their size is indicated in the table. After 10 weeks on the various diets, the animals were anesthetized with nembutal given intraperitoneally, heparin was injected into the jugular vein, and blood collected by carotid section. With this procedure it is quite simple to obtain 5 ml of blood from a 150-gm rat. Plasma obtained in this manner was then used for determinations of calcium, phosphorus, alkaline phosphatase and total proteins. These data are presented in table 2.

TABLE 2  
*The effects of caloric restriction.*

GROUP	NUMBER OF RATS	DIET	INITIAL WEIGHT	WEIGHT CHANGE	FOOD INTAKE	PLASMA CA	PLASMA P	PLASMA PHOSPHATASE	PLASMA PROTEINS
			gm	gm	gm/day	mg %	mg %	B.U. <sup>1</sup>	gm %
1	8	A	52	+168	9.6	9.1	7.3	15.2	7.2
2	8	D	53	+57	7.0	9.0	6.4	22.6	7.0
3	8	B	50	+3	5.0	8.7	6.1	28.4	6.6
4	8	C	53	-4	5.0	8.8	6.2	25.1	6.0
5	8	A	255	+82	16.3	9.2	6.8	12.4	7.4
6	8	B	251	-96	9.0	8.8	7.4	23.2	5.6
7 <sup>2</sup>	6	A	237	+48	13.4	7.3	7.8	9.0	6.8
8 <sup>2</sup>	6	B	241	-116	8.0	7.2	8.5	21.5	5.7

<sup>1</sup> Bodansky Units.

<sup>2</sup> Parathyroidectomized.

All animals were photographed by x-ray at the start of the experiment and again at its termination. Shown in figure 1 is a typical member of each group. From these plates it can be seen that the young rats, on a 50% caloric intake, not only failed in their generalized growth but also showed a complete cessation of skeletal growth as well. Considering also the fate of animals on the intermediate level of caloric intake, it appears that in young rats skeletal growth is roughly proportional to general body growth. The limited protein intake of group 4 did not affect this situation.

In contrast, considering the plates of the rats in groups 5 and 6, it appeared that in the larger rats, who were almost fully grown, a caloric restriction sufficient to produce a weight loss of 100 gm in 10 weeks (40% of their initial body weight) resulted in only slight interference with skeletal development.

Despite the impairment of lengthwise and transverse growth of the skeleton of caloric restricted young animals, no unusual changes were observed in the plasma calcium concentration in such animals. There did appear to be a definite tendency toward lowering of the serum inorganic phosphate concentration although this was not as marked as that seen in rickets and was not encountered in the larger animals. In contrast, in all series caloric restriction elicited a marked increase in the serum alkaline phosphatase activity.

Since identical increases in plasma phosphatase activity had been obtained in earlier series, it was then thought of interest to observe the effects of caloric restriction in parathyroidectomized rats. Because of the difficulties inherent in parathyroid ablation in rats, total thyroparathyroidectomies were performed under ether anesthesia. After a few days on a stock diet the rats were transferred to diets A and B to which 0.1% desiccated thyroid powder had been added. They are included in table 2 as groups 7 and 8. The animals on diet A did not grow quite as rapidly as did normal con-

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Figs. 1, 2 and 3 X-ray photographs of 1 typical member of each of the groups described in table 2. The photographs in the A series were taken on the first experimental day while those of the B series represent the same rats 10 weeks later. The photographs were taken under standardized conditions and developed and printed under identical conditions so that the figures reflect real differences in bone density.

1. Young rat, fed *ad libitum*.
2. Young rat, caloric intake  $\frac{1}{3}$  of that of group 1.
3. Young rat, caloric intake  $\frac{1}{2}$  of that of group 1.
4. Young rat, caloric intake and protein intake  $\frac{1}{2}$  of that of group 1.
5. Large rat, fed *ad libitum*.
6. Large rat, caloric restricted.
7. Parathyroidectomized large rat, fed *ad libitum*.
8. Parathyroidectomized large rat, caloric restricted.

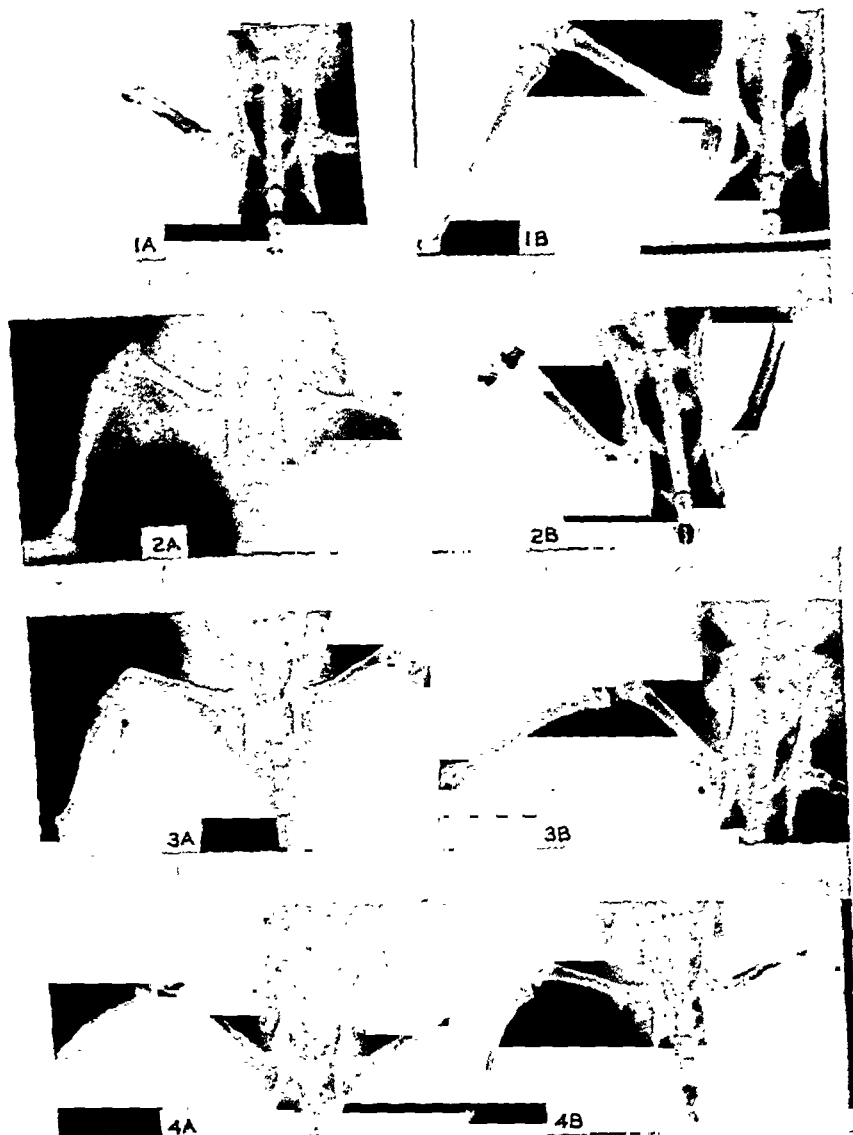


Figure 1

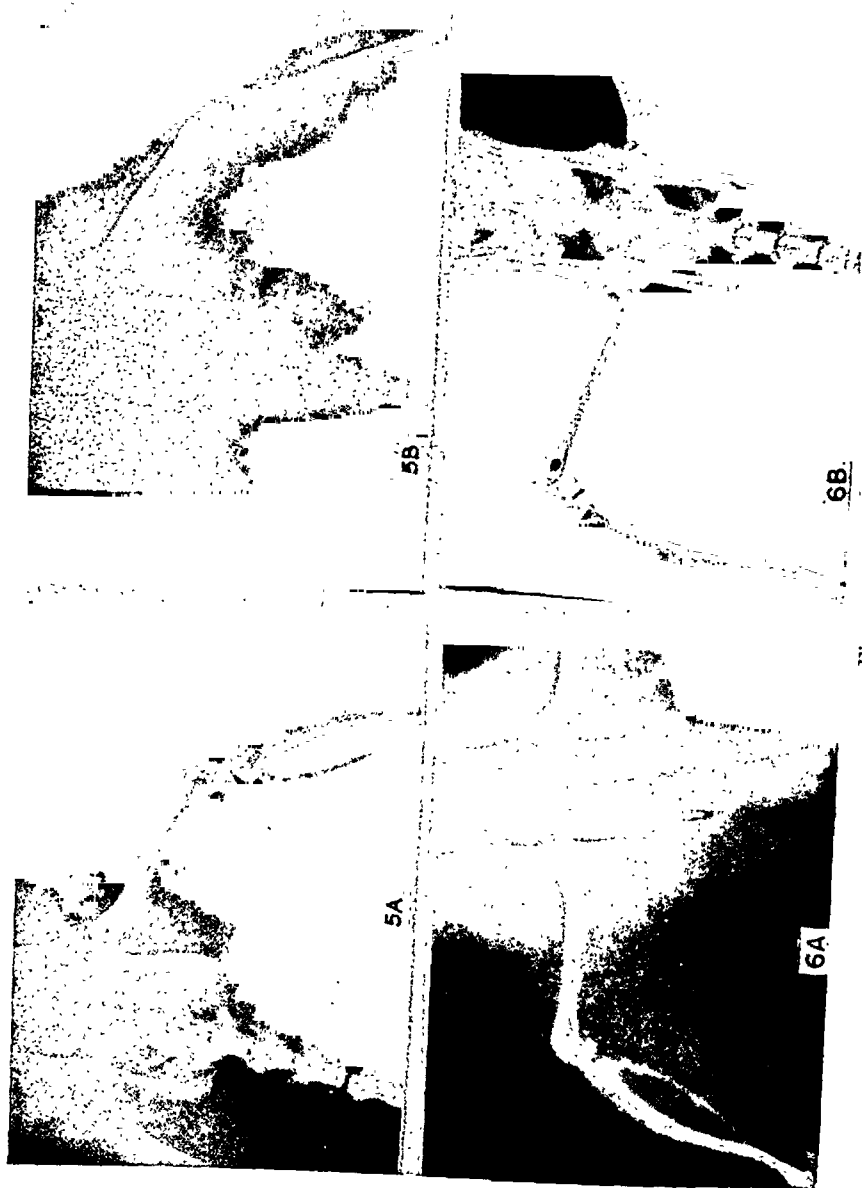


Figure 2



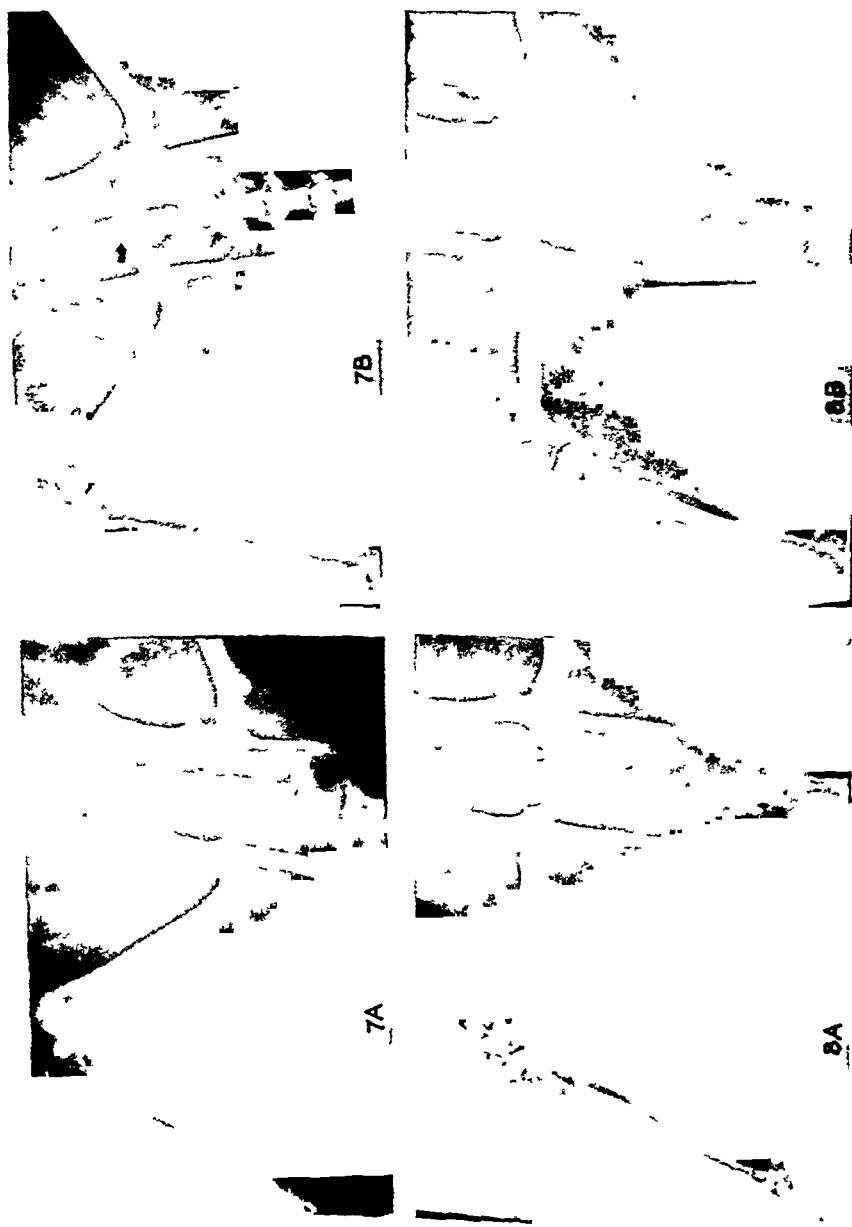


Figure 3

trols of this size while the animals on diet B lost somewhat more weight than did the unoperated animals in group 6. In all other respects, however, they resembled the unoperated animals. Like the rats in group 6 (caloric restricted large rats) the skeletons of the rats in group 8 continued to grow while the animals lost weight. As was to be expected they presented lower plasma calcium concentrations and higher phosphate concentrations than their controls. Of particular interest was the fact that in these parathyroidectomized, caloric restricted animals, also, there occurred a dramatic rise in serum alkaline phosphatase activity.

Representative femurs from rats in each group were fixed in formalin, and sections prepared for histological examination. In the younger animals there was found definite alteration in bone formation. There were fewer trabeculae; those that were present were stumper than normal and the cartilage plate at the epiphyses was not growing actively. In addition, the cortices appeared thinner than in the control animals. There was no excess osteoid. In the older animals similar differences were observed but in lesser degree. All these changes are non-specific and resemble what is encountered in inanition which has been produced by various means. In summary, these changes are those which are usually observed in poorly growing or athreptic animals.

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Fig. 4 X-ray photographs of the right, hind legs of typical members of each of the groups in table 2. The legs were dissected free and then photographed simultaneously on 1 plate. The numbers shown correspond to the group numbers of table 2, but the animals chosen are, in each case, different from those shown in figures 1-3.

1. Young rat, fed *ad libitum*.
2. Young rat, caloric intake  $\frac{2}{3}$  of that of group 1.
3. Young rat, caloric intake  $\frac{1}{2}$  of that of group 1.
4. Young rat, caloric intake and protein intake  $\frac{1}{3}$  of that of group 1.
5. Large rat, fed *ad libitum*.
6. Large rat, caloric restricted.
7. Parathyroidectomized large rat, fed *ad libitum*.
8. Parathyroidectomized large rat, caloric restricted.

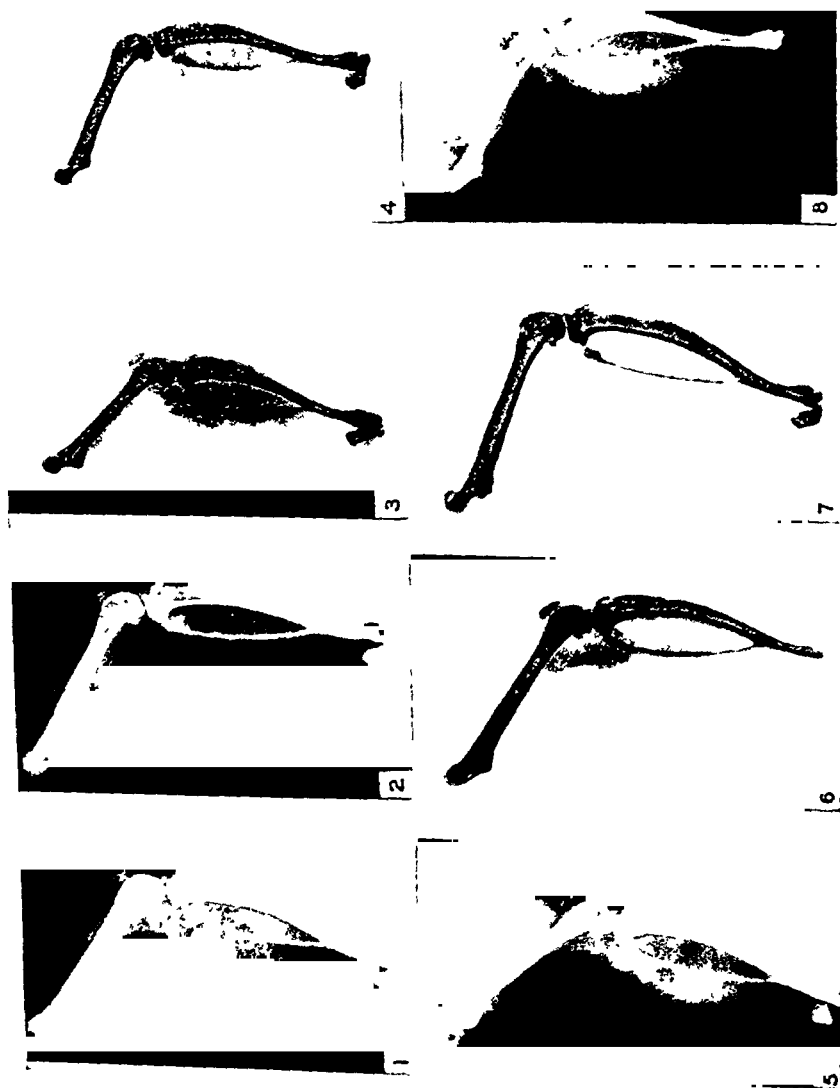


Figure 4

## DISCUSSION

From the data presented herein it seems quite clear that caloric restriction results in impairment of skeletal growth and that this phenomenon is more marked in young rats than in those whose growth is almost complete. Since growth and remodeling of bone are functions of age, this observation is not unexpected. Moreover, since skeletal growth, like that of any other tissue, is dependent upon a cellular mechanism which requires an active carbohydrate metabolism (Gutman, Warrick and Gutman, '42) a disturbance in osteogenesis is not surprising. In a sense, these experiments serve as a control for comparison with the numerous previous studies of the effects of partial or complete inanition and indicate that the possibility of inadequate caloric supply must be considered whenever bone growth is being studied, independent of the supply of those nutrients which have a specific effect on bone formation such as ascorbic acid, vitamins A and D, calcium and phosphorus. These experiments certainly indicate a need for pair-fed controls whenever studies are made of osteogenesis in nutritionally deficient animals. It may be presumed that the classical picture of bone in inanition is more likely to be the result of an inadequate caloric supply than of any other single dietary factor. It would be of interest to determine whether this picture might be modified by the action of estrogens, which decelerate the normal destruction of bony trabeculae just beneath the cartilage shaft junction (Day and Follis, '41), or androgens, since both of these can induce positive calcium balance, particularly in osteoporotics (Reifenstein and Albright, '47).

At present it is not possible to interpret the results of the blood chemical findings in these animals in a definitive fashion. The plasma of all young, caloric restricted animals was found to have an essentially normal calcium concentration but diminished inorganic phosphate concentration and markedly elevated alkaline phosphatase activity. The plasma inorganic phosphate concentration of caloric restricted animals of the older age group was not essentially different from that of

their controls. This may be correlated with the fact that skeletal growth proceeded at the normal slow rate in these animals. However, they too presented elevated plasma alkaline phosphatase activity. It would seem then that this rise in phosphatase activity may not have been a reflection of metabolic events in the skeleton, but, rather, was the result of an altered hepatic metabolism under these circumstances.

Investigators of the effect of total starvation on serum alkaline phosphatase activity (Bodansky and Jaffe, '31; Weil and Russell, '40) have consistently encountered a fall of about 50% from normal values. However, these studies were, perforce, of brief duration and Wachstein ('45) has found that the alkaline phosphatase activity of the livers of rats and mice dying of starvation, determined histologically, is decidedly elevated. Since Oppenheimer and Flock ('47) have found that the increased hepatic alkaline phosphatase activity during the regenerative period following partial hepatectomy in the rat is accompanied by an increased plasma phosphatase activity it would seem that such phosphatase can escape from the liver to accumulate in the circulation. This possibility seems all the more likely when one considers that there is no other evidence of failure of biliary concentration in partially hepatectomized rats and the alkaline phosphatase activity of both normal and regenerating liver is largely bound to the chromatin structure in the nucleus or mitotic figures. Moreover, although no evidence is available which demonstrates a direct control by the parathyroid of bone or serum alkaline phosphatase, it does seem strange that parathyroidectomy should not have affected the level of plasma phosphatase activity in caloric restricted rats if the phosphatase were arising in the metabolically deranged bone. Considering these facts, it is felt by the authors that while the low plasma phosphate and high alkaline phosphatase concentrations in young caloric restricted rats are accompanied by osteogenetic disturbances, they are probably the consequence of an altered hepatic carbohydrate metabolism rather than of metabolic events in the skeleton.

## SUMMARY

In young rats the rate of skeletal growth was roughly proportional to the rate of general body growth when caloric restriction was the limiting growth factor. Restriction of caloric intake to about one-half of that of *ad libitum* fed control animals, while ensuring an adequate supply of all other nutrients, resulted in a complete cessation of both skeletal and generalized body growth. Histologically the bones of such animals were identical with those usually found in "partial inanition." In larger, not quite completely grown rats, caloric restriction, sufficient to cause a loss of 40% of their initial body weight, only slightly decreased the rate of skeletal growth.

The plasma concentration of calcium was normal in all animals, while that of inorganic phosphate was below normal in the young caloric-restricted animals but not in the older rats. Caloric restriction in young rats, older rats and parathyroidectomized older rats resulted in a markedly elevated plasma alkaline phosphatase activity. However, it is thought that these latter changes in the caloric restricted rats are due to an altered hepatic carbohydrate metabolism rather than to the defective bone metabolism.

## ACKNOWLEDGMENTS

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# THE EFFECT OF ADDED THIAMINE ON GROWTH, VISION, AND LEARNING, USING IDENTICAL TWINS

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Two reports have been published (Harrell, '43 and '46) which indicated that when half of the children living in an orphanage were given 2 mg of thiamine daily, in addition to their meals, their vision and their ability in a considerable number of psychological tests improved as compared with the other half of the children who received placebos and served as controls. Each child in the control group was matched as closely as possible with one receiving thiamine. For 2 periods, several days in length, all the foods eaten in the orphanage were weighed and the thiamine intake per child was calculated from tables. The estimated intake varied from 0.9 to 1.0 mg per child per day, but it was noted that "no deduction was made for possible excessive cooking loss."

## PRESENT STUDY

In the present study, 44 pairs of identical or uniovular twins, living in their homes, were used as the test subjects. The twins were shown to be identical, that is, 1-egg pairs, by

<sup>1</sup> Swift Foundation research fellow.

the comparison of the color of their eyes and hair and by the study of their finger, palm and foot prints (this part of the study was carried out by Dr. Norma Ford Walker and Mrs. Reid Weaver). This method is accepted as reliable by geneticists. Identical twins provide subjects as closely matched as possible. After the initial examination was completed (see later), 1 member of each pair received 2 mg of thiamine and the other a placebo each day.<sup>2</sup> At the beginning of the study the children ranged from  $7\frac{1}{2}$  to  $15\frac{1}{2}$  years in age and were in grades 1 to 9 in school.

#### CALCULATION OF THIAMINE IN DIET

The eating habits of the 2 members of each pair were nearly always found to be closely similar when the mother and the individual children were questioned on this subject, on 3 or more occasions. In April and May, when the children had been receiving the tablets about 4 months, each mother was asked to keep a week's record of all food eaten by each of the twins. As the food was not weighed, the amounts were recorded in common household measures. A calculation of the thiamine content of the food eaten was then made, using the Table of Food Values recommended for use in Canada (Nutrition Division, Department of National Health and Welfare, '46). The results obtained are shown in table 1. Nine of the 24 pairs of twins were eating approximately the recommended daily allowances of thiamine (Food and Nutrition Board, National Research Council, '45), as is shown in column 3 of this table, headed "middle range." Twelve of the 24 pairs were eating 20 to 40 % less than the recommended allowances. In this case, the calculated intakes are shown in column 2, under the heading of "low." Three of the pairs were eating more than the recommended amounts (column 4). It was impossible, under the circumstances, to carry out the numerous determinations necessary to assay chemically the amount of thiamine in the food.

<sup>2</sup> Tablets donated by Mead Johnson and Co.

No correlation could be found between the estimate of the thiamine content of the diets and the effectiveness of the thiamine supplement, as measured by the tests listed below. The economic positions of the families varied greatly. Eight pairs were living in very poor homes and unfortunately 5 of these had to be discarded because the parents did not co-operate in giving the tablets regularly. Three other pairs, living in good homes, also had to be dropped for the same reason. Of the 36 pairs who took their tablets with sufficient regularity, 5 pairs were quite well-to-do, 28 pairs were apparently in fair to moderately good financial circumstances and 3 pairs were in poor homes.

TABLE 1

AGE IN YEARS	CALCULATED DAILY THIAMINE IN FOOD EATEN BY 24 PAIRS OF TWINS			AMOUNTS RECOM- MENDED BY FOOD AND NUTRITION BOARD, N. E. C.
	Low	Middle range	High	
	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>
8	0.7	0.9	2.0	1.0
9	0.7	0.8, 0.9		1.0
10	0.7	1.1, 1.1		1.2
11	0.8	1.0	1.3	1.2
12	0.9	1.1		1.2
13 (boys)	0.9, 0.9, 1.2		1.8	1.5
13 (girls)	1.0, 1.0	1.3		1.3
14 (boys)	0.9, 1.0			1.5
14 (girls)		1.3		1.3

#### PROCEDURE—INITIAL TESTS PRIOR TO EXPERIMENTAL PERIOD

Thanks to the kind co-operation of the school staffs, facilities were provided which made it possible to carry out all the tests in the schools. Initial tests, prior to the experimental period, were made in order to compare the physical and psychological status of the members of each pair and also to exclude any pair in which 1 twin was severely handicapped. These tests included a physical and neurological examination, the determination of weight (indoor clothes, no shoes), standing height (no shoes), a rough test of hearing and a careful test of the vision of each eye alone and of both eyes together.

For the latter a Snellen chart,<sup>3</sup> on which the illumination could be kept constant, was used. This apparatus included a light meter, set in a fixed position, which measured the light reflected from the chart and a rheostat for regulating the intensity of the light emitted by the 2 rows of frosted bulbs that were mounted on the right and left sides of the chart. Care was also taken to ensure good illumination in the room where the test was done. The child's vision was scored by allowing a specified mark for each letter read correctly at a distance of 20 feet — the marks becoming progressively larger as the letters became smaller.

The following 8 tests were given by the psychologist taking part in the study. They took 1 whole school day to administer, and in order to reduce the effect of fatigue, the time was spread over 2 days. For example, a pair of twins examined one morning would receive the remainder of the tests the following afternoon.

#### PSYCHOLOGICAL TESTS

NAME OF TEST	ABILITY TESTED
1. <i>Individual test of intelligence</i> Binet, form L	Intelligence
2. <i>Test of reasoning</i> , <sup>4</sup> <i>Grades 1-8</i> (Simple problems)	Reasoning
3. (a) <i>Dominion Test of Reading</i> Vocabulary (grades 2 and 3)	Reading
(b) <i>Gates Reading Survey</i> Vocabulary (grades 3-10)	Reading
4. (a) <i>Arithmetic achievement tests</i> <sup>5</sup> (For grade 2 and for grade 3)	Arithmetic
(b) <i>Dominion Arithmetic Test</i> Fundamental operations (grades 1-8)	Arithmetic
5. <i>Test of Rote Memory</i> <sup>6</sup> Memorizing meanings of 18 entirely strange words; testing immediately thereafter.	Immediate memory

<sup>3</sup> We are indebted to the Department of Physiology, University of Toronto, for the loan of this apparatus, which had previously been used in the Royal Canadian Navy.

<sup>4</sup> Prepared by Dr. John Long, Ontario College of Education.

<sup>5</sup> Prepared by Dr. A. J. Phillips, Ontario College of Education.

<sup>6</sup> Courtesy of Dr. Ruth F. Farrell, Norfolk, Virginia.

## NAME OF TEST

## ABILITY TESTED

6. *Testing of Faces and Names*<sup>4</sup>

Given 5 short measured times to memorize names of 20 faces during initial testing. Given no chance to refresh memory subsequently, but shown faces and asked to name them.

Retention or forgetting over prolonged period

7. *Test of Code Substitution*<sup>7</sup>

Code at the top of sheet consisting of 5 geometric symbols with a different digit under each. Child to put correct digits under symbols printed on large sheets in specified time.

Speed, accuracy

8. *Test of Nail Sorting*<sup>8</sup>

Filling the greatest number of standard holes with 3 nails, head up, in a given time.

Manual dexterity

## RESULTS OF INITIAL TESTS

As soon as the initial tests were completed, the senior author assigned one twin to the experimental group, to receive thiamine, and the other twin was placed in the control group. The psychologist, the twins, the parents and the teachers did not know which child was receiving the thiamine.

The average scores of the 2 groups at the beginning of the experimental period were very similar in all the tests. The results of a number of the tests are shown in table 2.

TABLE 2

*Averages of initial tests in 36 pairs of twins.*

	CONTROL	EXPERIMENTAL
Binet (I.Q. points)	104.6	103.6
Height (inches)	55.9	55.7
Weight (pounds)	73.5	77.4
Code substitution	201.4	201.3
Nail sorting	84.1	82.3

The intelligence quotients ranged from 76 to 158 points. The 2 individuals in 7 pairs had the same I. Q. (although not due to passing identical items), those in 16 pairs showed less than 5 points difference, and those in 13 pairs more than this.

As far as the individuals making up the pairs were concerned, their abilities in the special tests used varied much

<sup>7</sup> Obtained from Teachers College, Columbia University, New York.

<sup>8</sup> Obtained from the Department of Psychology, University of Toronto.

more than was anticipated. The psychological differences found in the pairs will be reported elsewhere.

#### EXPERIMENTAL PERIOD

##### *(A) Short term experiment — first 4½ months*

As soon as the initial tests were completed the twins were started on therapy. The placebos were made of lactose of identical shape and size as the thiamine-containing tablets, but were colored a pale pink. Each child had his own box of counted tablets, with his name clearly marked on it and a red seal as well in the case of the placebos. The importance of giving each twin his own tablet once a day, preferably after breakfast, was stressed by the senior author when the mother was visited, and she was given a calendar on which to mark each day on which tablets were given. The mothers were visited once a month by the senior author, when the number of tablets remaining was counted, the calendar checked, and more tablets left with her. The mothers, who were all very interested in their twins, were extremely cooperative. They had been told that 1 twin would receive more thiamine per day than the other. This, of course, included the thiamine eaten in their food. The short-term test covered an all-over testing period of 7 months for the examiners (November to June), and of 4½ months (the average figure) for each pair of twins. During this time, the average number of tablets forgotten was 7. Seven pairs of twins remembered to take the tablets every day and the maximum forgotten by any pair was 29. The psychological tests numbered 5, 6, 7 and 8 were re-administered every 3 weeks during this 4½ months of therapy. At the end of the short-term test, the whole battery of physical and psychological tests were repeated. At this time, form M of the Binet test was used, and the alternative forms of tests 2, 3 and 4.

##### *Results of short-term test (first 4½ months)*

These results are shown in table 3. The scores obtained were subjected to statistical analysis. When 2 trials only had

been given, the level of significance of the superior or inferior gains made by the thiamine-fed children was measured by the t-statistic. Where more than 2 trials were given, the method of analysis of variance was used. The latter provides an F-statistic for the significance of the differences between the trends of the scores of the experimental and the control

TABLE 3

*Superior or inferior gains of thiamine-supplemented children in short-term test.*

Subjects — 36 pairs                      Duration — 136 days (average)

F — Statistics and t — Statistics with level of significance.

NAME OF TEST	T <sub>0</sub>	LEVEL OF SIGNIFICANCE OF T <sub>0</sub>	F <sub>1</sub>	LEVEL OF SIGNIFICANCE OF F <sub>1</sub> — 5%
Intelligence — Binet	— 0.772	40%		
Reasoning — (Gr. 4-8)	0.199	80%		
Reading — vocabulary (Gr. 2)	— 0.612	60%		
Reading — vocabulary (Gr. 3-10)	— 0.073	90%		
Arithmetic (Gr. 2 and 3)	— 1.498	20%		
Arithmetic (Gr. 4-8)	— 1.596	10%		
Rote memory			0.773	2.43
Code substitution			0.462	2.43
Manual dexterity			2.336	2.43
Faces and names (retention)			2.159	3.09
Height	2.187	5%		
Weight	2.260	Between 2 and 5%		
Eyesight (left eye)	0.925	40%		
Eyesight (right eye)	1.108	30%		
Eyesight (both eyes)	0.902	40%		

— Indicates tests in which control group made superior gains, as compared with thiamine-supplemented group.

groups. (F<sub>1</sub> indicates the significance of differences between trials.)

From table 3 it is seen that the experimental group (receiving thiamine) made superior gains in height (5% level) and weight (between 2% and 5% level). This means that in only 5, or 2 times, as the case may be, out of 100 could these

superior gains be attributed to chance. The 5% level of significance is not considered conclusive but only doubtful. In the tests of Nail Sorting (manual dexterity) and Faces and Names (retention or forgetting over a prolonged period), the experimental group made superior gains which almost reached the 5% level of significance. In reasoning, rote memory, code substitution, and eye-sight, the superiority of the experimental group was very slight and not significant statistically. In intelligence, reading, and arithmetic, the control group was slightly superior but this again was not significant statistically.

*(B) Long-term test (9 months' duration)*

The long-term test included the short-term one described above plus an extension, over the summer holidays, of roughly 4½ months. During the summer it was not possible to maintain close contact with the families and as a result 11 pairs of twins stopped taking the tablets or took them very irregularly. The long-term study, therefore, comprised 25 pairs of twins. During the 9 months of this test, the average number of tablets missed was 10, the maximum number forgotten was 29, and 2 pairs took them every day. At the end of the 9 months, the complete battery of physical and psychological tests was readministered, using the original forms. The results are shown in table 4.

It is seen that in reading and arithmetic the older children (grades 3 to 10) in the control group made slightly greater gains than those given the thiamine supplement. These gains were not significant statistically. In the other tests, the experimental group (thiamine-supplemented) showed slightly greater gains, which again were not significant statistically. Why the control group largely "caught up" on the thiamine-supplemented group, during the summer, is not clear. It has been reported that American children grow more in weight during the summer and autumn than in the rest of the year. Also their diets, during the summer and autumn, contain more fruits and vegetables, which would increase the thiamine in-



take to some extent. Previous investigators (Ross and Summerfeldt, '35), have shown that the addition of vitamin B complex improves the growth of children from 5 to 14 years of age.

TABLE 4

*Superior or inferior gains of thiamine-supplemented children in long term test.*

Subjects—25 pairs                      Duration—273 days (average)  
F—Statistics with levels of significance.

NAME OF TEST	F <sub>1</sub>	LEVEL OF SIGNIFICANCE OF F <sub>1</sub> —5%
Intelligence—Binet	1.613	3.19
Reasoning (Gr. 4-8)	1.073	3.26
Reading—vocabulary (Gr. 3-10)	—2.62	3.25
Reading—vocabulary (Gr. 2)	0.51	4.46
Arithmetic—(Gr. 4-8)	—1.75	3.26
Arithmetic—(Gr. 2 and 3)	0.48	4.10
Rote memory	0.529	2.29
Code substitute	0.624	2.29
Manual dexterity	0.345	2.29
Faces and names (retention)	1.203	2.46
Height	0.483	3.19
Weight	1.148	3.19
Eyesight (left eye)	0.83	3.20
Eyesight (right eye)	1.39	3.21
Eyesight (both eyes)	0.61	3.20

— Indicates tests in which control group made superior gains, as compared with thiamine-supplemented group.

#### CONCLUSIONS

1. The administration of 2mg daily of thiamine for 4½ months to half of 36 pairs of identical twins resulted in improvement in weight and height gains, and in manual dexterity and prolonged memory test scores, that were of questionable statistical significance. The tests used for measuring vision, intelligence, reasoning, arithmetic, rote memory and code substitution gave results of no statistical significance.

2. Twenty-five pairs of the twins were kept in the study for 4½ months longer. When they were again tested at the end

of this period, that is, after 9 months of therapy, no gains of statistical significance were evident in any of the tests.

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# STUDIES ON THE REQUIREMENT OF THE CHICK FOR TRYPTOPHANE

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ONE FIGURE

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Since the avian species' diet consists largely of cereal grains and their by-products, and since the limited amount of tryptophane present in most cereal grains is recognized, the need for considerable information on the tryptophane requirement by various members of the avian species is emphasized.

Work on the tryptophane requirement of the chick has been reported by Almquist and Mecchi ('41) and Grau and Almquist ('44). Recent studies by Briggs ('45) and Briggs, Groschke and Lillie ('46) demonstrated that the tryptophane requirement was influenced by the level of nicotinic acid present in the ration.

In the present work, experiments were conducted to obtain additional information on the tryptophane requirement of the chick when optimum levels of nicotinic acid were supplied in the ration. An improved basal ration was developed in which the protein was supplied by oxidized casein (Toennies, '42) and gelatin supplemented with the sulfur amino acids. This ration when properly supplemented with tryptophane supported growth which was comparable to that obtained with a practical chick starter or with diets containing untreated

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TABLE 1

*The amino acid composition of the basal ration and the reported requirement of each amino acid for the chick.*

AMINO ACID <sup>1</sup>	AMINO ACID SOURCES			TOTAL AMINO ACID CONTENT	REPORTED REQUIREMENT AND REFERENCE
	12% oxidized casein	10% gelatin	2% fish solubles (dry basis)		
	gm	gm	gm	%	%
Histidine	0.34	0.06	0.03	0.48	0.15 <sup>2</sup>
Arginine	0.47	0.91	0.06	1.44	1.0 <sup>3</sup> ; 1.2 <sup>2</sup>
Lysine	0.92	0.58	0.06	1.56	0.9 <sup>3</sup> ; 1.1 <sup>2</sup>
Leucine	1.19	0.35	0.06	1.60	1.5 <sup>4</sup>
Isoleucine	0.67	0.17	0.04	0.88	0.5 <sup>4</sup>
Valine	0.80	0.27	0.04	1.11	0.7 <sup>4</sup>
Methionine	0.00	0.06	0.02	0.08	0.6 <sup>3</sup> ; 0.5 <sup>2</sup>
Threonine	0.44	0.20	0.03	0.67	1.0 <sup>2</sup>
Tryptophane	0.003	0.001	0.004	0.008	0.5 <sup>3</sup> ; 0.25 <sup>2</sup>
Phenylalanine	0.71	0.23	0.03	0.97	0.5 <sup>2</sup>
Glycine	0.06	2.36	0.09	2.51	1.0 <sup>3</sup> ; 1.8 <sup>2</sup>
Cystine <sup>5</sup>	0.016	0.010	0.008	0.034	0.4 <sup>2</sup>

<sup>1</sup> The amount of tryptophane and methionine in the oxidized casein was determined in this laboratory; all other values were taken from either Toennies ('42), Block and Bolling ('45), Stokes et al. ('45), or Lassen and Bacon ('46).

<sup>2</sup> Almquist and Grau ('44).

<sup>3</sup> Cravens, Almquist, Norris, Bethke and Titus ('44).

<sup>4</sup> Grau and Peterson ('46).

<sup>5</sup> Grau and Almquist ('44).

<sup>6</sup> Not an essential amino acid

TABLE 2

*Comparison of the growth promoting properties of several rations.*

CHICKS USED	WHITE LEGHORN <sup>1</sup>			NEW HAMPSHIRE X WHITE LEGHORN <sup>1</sup>		
	dl tryptophane added	Number chicks	Gain per chick	dl tryptophane added	Number chicks	Gain per chick
Basal diet	%		gm	%		gm
12% oxidized casein	0.8	10	15	0.6	10	144
12% casein	0.5 <sup>2</sup>	8	19	0.34 <sup>2</sup>	10	149
18% oxidized casein	0.8	9	15			
18% casein	0.45 <sup>2</sup>	8	16			
Practical chick starter		8	16		10	148

<sup>1</sup> Day old White Leghorn chicks were fed the experimental diets for 8 days. The crossbred chicks were placed on experiment at 10 days of age. Experiment conducted for 14 days.

<sup>2</sup> The l( )tryptophane contents of the casein diets were adjusted to approximately equal those of the oxidized diets.

basis for the second method for the determination of the tryptophane requirement of the chick. The technique used in this method involved the substitution of casein for oxidized casein in the basal ration and the feeding of graded levels of casein as the source of tryptophane. The casein added in excess of 12% was supplied at the expense of the starch.

Since the tryptophane requirement of the chick is influenced by the amount of nicotinic acid in the ration, 5 and 10 mg of nicotinic acid per 100 gm of ration were fed with a sub-optimum level of tryptophane. The growth response was the same for both groups thereby indicating that, for the ration used, 5 mg nicotinic acid per 100 gm of ration supplied an adequate quantity of this dietary constituent, and this level of nicotinic acid was used in all subsequent experiments.

*Studies with graded levels of tryptophane.* It had been previously reported that the requirement for tryptophane in the *dl* form was twice that for the natural form, and that the chick required approximately 0.25% *l*(-)-tryptophane in the diet (Grau and Almquist, '44). Therefore the first experiment in this series was designed to feed graded levels of *dl*-tryptophane<sup>3</sup> ranging above and below the recommended level. The crossbred chicks were used in this experiment. The chicks receiving the basal diet to which no tryptophane had been added lost 18 gm (table 3). Approximately 70% of this loss in weight occurred during the first 5 days. The deficient chicks were less active and they remained inside the heated portion of the brooder most of the time. The primary wing feathers were ragged, comb growth was retarded and the chicks appeared weak and emaciated. Typical birds fed the tryptophane deficient and tryptophane supplemented ration are shown in figure 1.

An analysis of variance showed no significant difference in growth between groups fed either 0.30, 0.40, 0.50, or 0.60% *dl*-tryptophane. The chicks fed these diets gained 9 to 10 gm per day, which is considered excellent growth, and the maxi-

<sup>3</sup> We are indebted to The Dow Chemical Company for generous gifts of *dl*-tryptophane.

TABLE 3

*Effect of feeding graded levels of tryptophane and casein on growth and efficiency of gain.*

	NUMBER CHICKS	WEIGHT AT 24 DAYS	GAIN PER CHICK <sup>1</sup>	GAIN PER GM FEED CONSUMED
		<i>gm</i>	<i>gm</i>	<i>gm</i>
Studies with tryptophane (New Hampshire × White Leghorn chicks)				
Tryptophane added to basal ration (%)				
<i>Experiment 1</i>				
None	10	76	18	— 0.11
0.30 dl	10	219	124	0.12
0.40 dl	10	229	135	0.17
0.50 dl	10	224	130	0.16
0.60 dl	10	238	144	0.18
<i>Experiment 2</i>				
None	10	66	— 17	
0.10 l( )	10	102	20	0.13
0.15 l(-)	10	194	112	0.38
0.20 l(-)	10	223	141	0.49
0.30 dl	10	225	143	0.50
0.40 dl	10	221	139	0.46
<i>Experiment 3</i>				
0.125 l(-)	10	108	27	0.15
0.175 l(-)	10	213	133	0.48
0.250 l(-)	10	204	123	0.43
0.30 dl	10	201	121	0.44
0.50 dl	10	208	128	0.44
Casein added to basal ration (%)				
Studies with casein				
New Hampshire × White Leghorn				
12	10	191	110	0.44
15	10	231	171	0.54
18	10	235	154	0.56
White Leghorn				
12	10	182 <sup>2</sup>	92 <sup>2</sup>	0.42
15	10	186	96	0.48
18	10	200	110	0.49

<sup>1</sup> Chicks were fed the experimental diet for 14 days.

<sup>2</sup> Average weight at 22 days.

<sup>3</sup> Chicks were fed the experimental diet for 12 days.

mal efficiency of gain obtained was 0.48. In terms of the reported tryptophane requirement, this was interpreted to mean either that the reported requirement was too high or that the chick was capable of utilizing the *d*-isomer of tryptophane or both. With the use of these experimental techniques, detailed



Fig. 1 New Hampshire  $\times$  White Leghorn chicks after 14 days on experiment. The chick on the left received an adequate diet and weighed 222 gm, while the chick on the right received the basal diet and weighed 63 gm.

data have been obtained on the utilization of the *d*-isomer of tryptophane by the chick and the utilization observed ranged from 17 to 40% (Wilkening and Schweigert, '47).

Experiment 1 indicated that 0.30% *dl*-tryptophane may supply adequate tryptophane to meet the chicks' requirement,

therefore experiment 2 was designed to expand and further investigate the findings of the first experiment. Crossbred chicks were fed graded levels of *l*(-)tryptophane ranging from none to 0.20%. Diets containing 0.30 and 0.40% *dl*-tryptophane were fed as the positive controls. The results of growth and efficiency of gain for the second experiment are presented in table 3.

The ration which contained 0.20 *l*(-)tryptophane supported growth comparable to that obtained with rations supplemented with 0.30 or 0.40% *dl*-tryptophane. It was obvious that the rations containing 0.0 and 0.10% *l*(-)tryptophane did not support growth comparable to rations containing the higher levels of tryptophane. A statistical analysis of the data showed that there was no significant difference in the growth of chicks fed diets containing 0.20% *l*(-)tryptophane and 0.30 and 0.40% *dl*-tryptophane. The gains observed with the 0.15% *l*(-)tryptophane ration were significantly different from those obtained with the 0.20% *l*(-)tryptophane ration ( $P < .02$ ). Thus it was shown that 0.20% *l*(-)tryptophane and 0.30 and 0.40% *dl*-tryptophane supplied an adequate quantity of tryptophane to support both optimum gains and efficiency of gain.

A third experiment was then conducted to further extend and confirm the results obtained in the first 2 experiments. Rations containing 0.125, 0.175, and 0.250% *l*(-)tryptophane and 0.30 and 0.50% *dl*-tryptophane were fed to the crossbred chicks. The results of this experiment are also shown in table 3. In the second experiment it was shown that the 0.15% *l*(-)tryptophane diet did not support maximal growth or efficiency of gain, but the 0.20% diet did. In this experiment, it can be seen that the 0.175% *l*(-)tryptophane diet supplied sufficient tryptophane to the basal ration for optimum growth or efficiency of gain. As was true in the previous experiments for rations supplemented with *dl*-tryptophane, the 0.30% diet supported maximal gains and efficiency of gain.

The growth data obtained with rations containing 0.175 and 0.25% *l*(-) tryptophane and 0.30 and 0.50% *dl*-tryptophane



were treated statistically. The F test showed no significant difference between any of these levels of *l*(-) or *dl*-tryptophane. Therefore, it was concluded that maximal rates of gain were obtained with diets containing either 0.175% *l*(-) tryptophane or 0.30% *dl*-tryptophane and that higher levels of tryptophane did not result in an increased growth response.

For maximal gain the minimum amount of *l*(-)tryptophane which was added to the basal ration was 0.175%. The basal

TABLE 4

*Effect of feeding graded levels of tryptophane on growth and efficiency of gain (White Leghorn).*

TRYPTOPHANE ADDED TO BASAL RATION	NUMBER CHICKS	WEIGHT AT 22 DAYS	GAIN PER CHICK <sup>1</sup>	GAIN PER GM FEED CONSUMED
%		gm	gm	gm
None	10	77	— 12	— 0.14
0.100 <i>l</i> (-)	10	107	18	0.15
0.135 <i>l</i> (-)	10	156	66	0.33
0.175 <i>l</i> (-)	10	178	88	0.44
0.200 <i>l</i> (-)	10	181	91	0.38
0.225 <i>l</i> (-)	10	184	94	0.44
0.30 <i>dl</i>	10	179	89	0.42

<sup>1</sup> The experiments were conducted for 12 days.

ration contained 0.008% *l*(-)tryptophane (table 1). Therefore the minimum level of *l*(-)tryptophane in a diet which supported optimum growth was 0.183%. From the results obtained with the oxidized casein diets, the *l*(-)tryptophane requirement of the New Hampshire × White Leghorn cross was therefore shown to be 0.18% of the diet.

Since the results obtained in this laboratory with respect to the tryptophane requirement of the chick were not in agreement with those reported by Gran and Almquist, whose experiments were conducted with the White Leghorn chick, it appeared desirable to obtain information on the White Leghorn. These workers used a 12-day feeding period, and the experiments performed in this laboratory with the Leghorn were conducted for a similar period of time. The results of this experiment are presented in table 4. While the rate of

gain was less for the White Leghorn, the relative response of the Leghorn to the graded levels of tryptophane was the same as that obtained with the hybrid chick. The growths obtained with rations containing 0.175, 0.20, and 0.225% *l*(-) tryptophane and 0.30% *dl*-tryptophane were all of the same order of magnitude, but the ingestion of rations containing lower amounts of tryptophane resulted in correspondingly less growth. There was no statistically significant difference in the gains obtained with rations containing 0.175, 0.20, and 0.225% *l*(-)tryptophane and 0.30% *dl*-tryptophane. These results are in agreement with those obtained with the crossbred chicks and thereby confirm the requirement of the chick for tryptophane at 0.18% of the ration.

Comparative blood studies were made on normal and deficient hybrid chicks from Experiment 2 which had been on the test for 14 days. In this study determinations were made on the hemoglobin and plasma protein levels and the apparent free tryptophane content of the plasma. The results obtained for hemoglobin and total plasma protein showed no difference between the normal and deficient groups. It appears, therefore, that a longer time would be required for a significant reduction to occur in these blood constituents. However, the apparent free tryptophane levels, determined microbiologically (Schweigert et al., '46) varied with the intake of tryptophane. The average content of apparent free tryptophane in the plasma of the group receiving the basal ration was 1.8  $\mu$ g per ml, while the average level for the group receiving an adequate amount of tryptophane was 6.5  $\mu$ g per ml of plasma. This observation confirms previous findings with the rat (Schweigert et al., '46).

*Studies with graded levels of casein.* Since the preliminary experiments indicated that diets containing untreated casein afforded a second method for determining the tryptophane requirement of the chick, an experiment was conducted using diets in which casein<sup>4</sup> replaced oxidized casein in the basal

<sup>4</sup>The casein used in these experiments analyzed 14.3% total nitrogen, 10.2% moisture, and 1.14 *l*(-)tryptophane. The values for total nitrogen and *l*(-)tryptophane are uncorrected.

ration. The casein was essentially the sole source of tryptophane in the diet, and the tryptophane content of the ration was varied by feeding graded levels of casein. The New Hampshire  $\times$  White Leghorn cross and the White Leghorn chicks were used in this experiment. The results are presented in table 3. In the experiment with the hybrid chick, the 12% casein diet produced neither optimum gains nor efficiency of gain. It was apparent that both the 15 and 18% casein diets produced a similar response, and statistically there was no significant difference between these groups. The difference in the growth obtained between the 12% and 15% casein diets was highly significant ( $P = 0.0012$ ).

The growth of the White Leghorn on the 15% casein diet was not comparable with that of the group fed the 18% casein ration, but the efficiency of gain was approximately the same for both groups. The group fed 15% casein consumed approximately 215 gm less feed than did either the group fed 12% or 18% casein. There was no available explanation for this decrease in feed consumption. Since the efficiency of gain had reached a maximum for both the White Leghorn and the hybrid and growth was at a maximum for the latter it was concluded that 15% casein supplied a quantity of tryptophane sufficient to meet the chicks' requirement.

The tryptophane content of the casein was determined by microbiological analysis using *Streptococcus faecalis* R as the test organism. The tryptophane was liberated from the casein by alkaline hydrolysis (Kuiken and Lyman, '47). The basal medium used for the determination of the tryptophane was the same as that used by Greenhut, Schweigert and Elvehjem ('46). The *l*(-)-tryptophane value obtained for the casein was 1.14%.

It was demonstrated that the 15% casein diet supplied an adequate quantity of tryptophane to meet the chick's requirement and that the casein contained 1.14% *l*(-)-tryptophane. Therefore, the 15% casein supplied 0.171% *l*(-)-tryptophane while the remainder of the ration supplied 0.005% *l*(-)-tryptophane. The total amount of *l*(-)-tryptophane contributed by

this ration was 0.176%. Consequently, the tryptophane requirement of the chick as determined with the casein diets was 0.18% of the diet.

The results obtained with the oxidized casein and casein diets are in excellent agreement. The *l*(-)tryptophane requirement of the chick, as determined by 2 independent methods with both the New Hampshire  $\times$  White Leghorn cross and the White Leghorn, is 0.18% of the diet for either growth or for efficiency of feed utilization.

There is no obvious explanation for the difference in the results obtained in this laboratory and those reported by Grau and Almquist. However, the level of nicotinic acid or other dietary components used may have been contributing factors.

#### SUMMARY

Chicks were fed a purified, tryptophane-low diet in which the major sources of protein were 12% oxidized casein and 10% gelatin supplemented with cystine and methionine. Chicks grew normally when an adequate amount of tryptophane was added to this diet. Using this ration with graded levels of tryptophane and a ration in which untreated casein supplied the tryptophane, the *l*(-)tryptophane requirement of the New Hampshire  $\times$  White Leghorn crossbred chick and the White Leghorn chick was shown to be 0.18% of the diet.

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# NUTRITIONAL STUDIES ON MILK FAT

## III. THE EFFECT OF THE TREATMENT OF MILK FAT WITH CERTAIN SOLVENTS ON THE GROWTH OF YOUNG RATS

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FIVE FIGURES

(Received for publication August 4, 1947)

Previous experiments (Jack et al., '45) have shown that young rats fed diets containing milk fat which had previously been dissolved in pentane (Skelly-solve A) did not grow as well as those fed diets containing untreated milk fat. Kratzer ('45) noted that the use of chloroform as a solvent had a similar effect on chicks. Examination of the fat which had been dissolved in pentane showed that it was not oxidized by the treatment, but was less resistant to oxidation as measured by the development of peroxides in accelerated oxidative stability tests at 80°C.

It seemed probable that the retardation of growth could have been caused by a destabilizing effect of the solvent either through chemical reaction or by a physical reorientation of the natural antioxidants present. The possibility of an impurity in the solvent which could have caused retardation of growth was not overlooked, although this was not considered likely since the solvent had a negative peroxide test.

### EXPERIMENTAL

A series of diets containing milk fat which had been dissolved in various solvents, followed by solvent-removal before feeding, was prepared and fed as described earlier (Hender-

son et al., '45). Two male and 2 female rats weighing about 45 gm at weaning were used in each lot. The solvents chosen were methyl alcohol, ethyl ether, acetone, chloroform, and pentane as representing the most common types of compounds usually used as fat solvents. The solvent was removed by distillation and subsequent sweeping with nitrogen to remove the last traces.

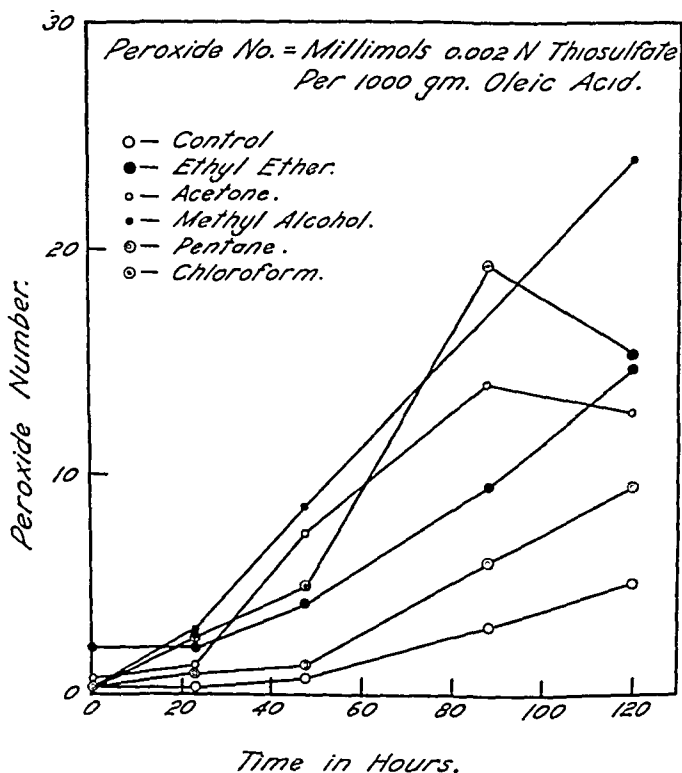


Fig. 1 The development of peroxides in milk fat treated with different solvents.

Figure 1 shows the oxidative stability of the treated fats. The acetone and chloroform were redistilled and ethyl ether was freed from peroxides over metallic sodium. Pentane does not show as great a destabilizing effect as the other solvents, but it should be essentially inert since it is a saturated hydrocarbon.



Figure 2 shows the growth of rats fed diets containing 20% milk fat treated with the previously mentioned solvents. This is a representative trial among several that were run, all showing essentially the same relationships. Pentane retarded

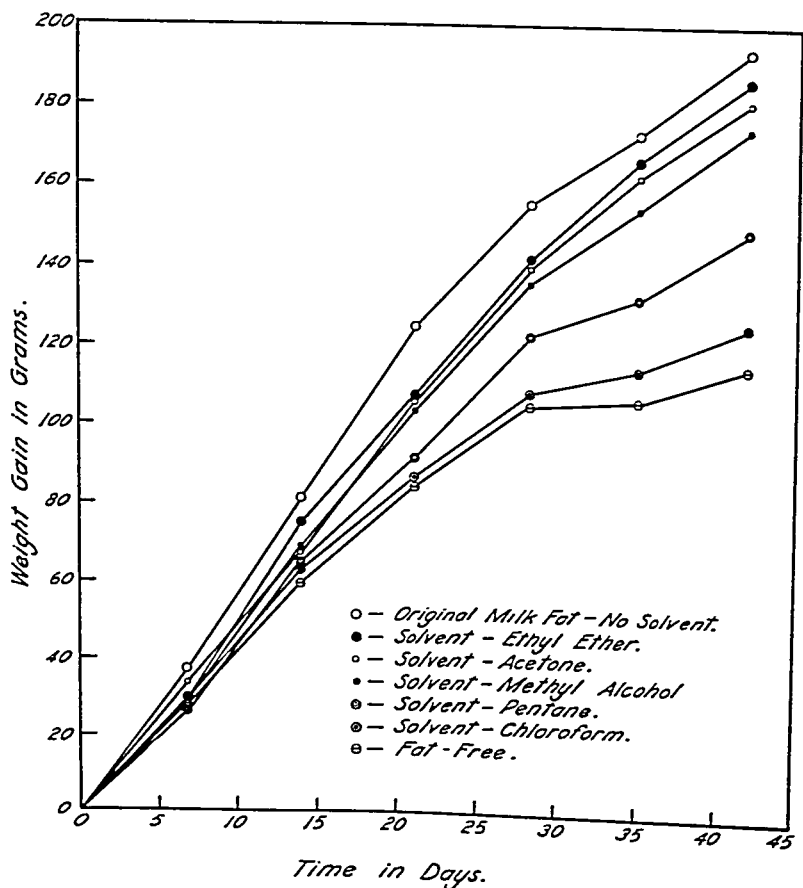


Fig. 2 The growth of young rats fed diets containing fats previously treated with different solvents.

growth to a greater degree than it reduced the oxidative stability and this led to an examination of the solvent for possible unsuspected impurities. Methyl alcohol caused the greatest increase in peroxide number but depressed growth very little. Ethyl ether did not depress growth but produced a high

peroxide number. There was, therefore, very little correlation between growth and peroxide number.

Sulphur is a common impurity in solvents of petroleum derivation but no evidence of sulphur could be found. A slight gummy residue that developed upon drying was suspected of being olefinic in character. Treatment with fuming sulphuric acid removed this residue and following this discovery, all pentane, except where so designated, has been treated with fuming sulphuric acid.

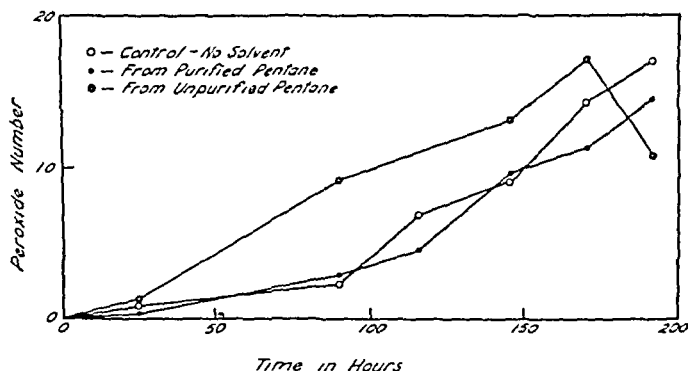


Fig. 3 The development of peroxides in milk fat as affected by purification of pentane.

Figure 3 shows the oxidative stability of milk fat which has previously been dissolved in purified and unpurified pentane. It is apparent that purification of pentane by treatment with fuming sulphuric acid removed the material responsible for reducing the oxidative stability.

Figure 4 shows the growth of rats on diets containing milk fat that had been dissolved in purified and unpurified pentane and also the growth on diets containing 3 milk fat fractions from purified pentane, prepared as described by Henderson and Jack ('44). Apparently, the deleterious effects of the impurities were removed since the fat which had been dissolved in the purified pentane produced the same growth as that which had not been dissolved in solvent.

In the previous experiments (Jack et al., '45) it was reported that milk fat, not solvent treated, produced slightly greater growth than the  $-53^{\circ}$  filtrate fraction. It will be noted that the  $-53^{\circ}$  filtrate used here from purified pentane produced growth substantially greater than the original fat.

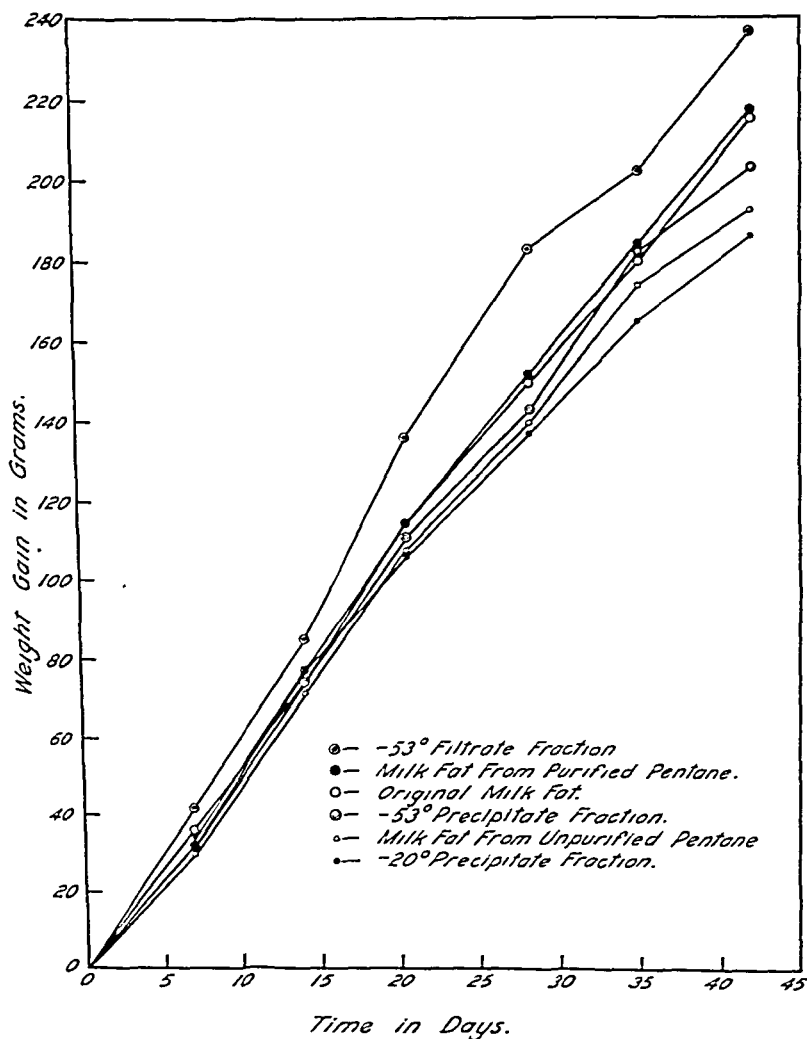


Fig. 4 The growth of young rats fed diets containing fat from purified and unpurified pentane.

This was to be expected since the  $-53^{\circ}$  filtrate used previously was prepared with unpurified pentane.

Table 1 gives the fatty acid composition of the  $-53^{\circ}$  filtrate fraction used in this experiment, together with the composition of that used previously for comparison.

TABLE 1  
*Fatty acid composition of  $-53^{\circ}$  filtrate fractions.*

LENGTH OF CARBON CHAIN	MOL PER CENT	
	From unpurified pentane <sup>1</sup>	From purified pentane <sup>2</sup>
	I.V. <sup>3</sup> 58.37, Sap. no. 236.9	I.V. 60.8, Sap. no. 235.4
Saturated		
C <sub>4</sub>	9.3	4.2
C <sub>6</sub>	6.9	1.6
C <sub>8</sub>	2.4	3.7
C <sub>10</sub>	4.5	1.1
C <sub>12</sub>	3.7	2.4
C <sub>14</sub>	7.4	5.4
C <sub>16</sub>	14.0	11.8
C <sub>18</sub>	6.8	9.2
C <sub>20</sub>	1.4	
Unsaturated		
C <sub>10</sub>	1.4	0.3
C <sub>12</sub>	0.4	1.1
C <sub>14</sub>	1.8	1.1
C <sub>16</sub>	4.6	11.7
C <sub>18</sub>	30.0	32.4
C <sub>20</sub>	0.8	4.9
C <sub>22</sub>		0.7
Linoleic	6.0	8.4

<sup>1</sup> Jack et al. ('45).

<sup>2</sup> This study.

<sup>3</sup> I.V. is iodine value; Sap. is saponification number.

The present  $-53^{\circ}$  filtrate fraction is slightly more unsaturated than the one previously used but otherwise is not greatly different in composition.

Table 2 shows the feed consumption and efficiency of utilization of feed on the diets containing the milk fat fractions prepared from purified pentane. These values are for the first 5 weeks only. The values for the entire 6 weeks for all diets

are not available because of failure of the attendant to weigh the remaining feed for some of the animals at the end of the trial. The data available indicate that there is no significant difference in 6 weeks from the 5-weeks' results.

It will be noted that the total feed consumption is not greatly different on the different diets. This is in accord with the previous findings by Jack et al. ('45) when milk-fat fractions were fed. The animals on the diet containing —53° filtrate fraction ate the least feed and gained the most weight. The animals on the diet containing the —20° ppt. fraction required the largest amount of feed per gram of gain.

The reports by Burr and his associates ('43, '44) concerning the deleterious effects of oxidized fat in the diet and the

TABLE 2

*Feed consumption and efficiency of feed utilization on milk fat fractions from purified pentane.*

DIET	FEED CONSUMED 5 WEEKS	GAIN IN WEIGHT 5 WEEKS	GRAMS FEED PER GRAM OF GAIN
Original fat	449	181	2.49
Original fat (Purified pentane)	444	184	2.41
Original fat (Unpurified pentane)	467	175	2.67
—20° ppt.	474	166	2.85
—53° ppt.	488	193	2.67
—53° Filtrate	441	203	2.18

stabilizing effects of tocopherols, taken in conjunction with the data presented here, suggested a possible nutritional significance in the oxidative stability of fat. A series of diets was prepared in which milk fat was used at different levels of oxidative stability as altered in the laboratory.

Milk fat unoxidized and with no peroxide number was used in the control. A portion of this fat was carried to the end of its natural induction period, to a peroxide number of 1–2. Another portion was mildly oxidized by bubbling oxygen through it to a peroxide number of 10–12. And a fourth sample was fortified with 0.15% of mixed tocopherols after being taken to the end of its natural induction period. These

fats were mixed in diets as before at a level of 20% and fed to young rats. These results are shown in figure 5.

The growth differences are small; however, the mildly oxidized fat did not produce quite as good growth as the other samples containing fat. Neither reducing the induction period nor fortifying the destabilized fat with tocopherol had any significant effect on growth.

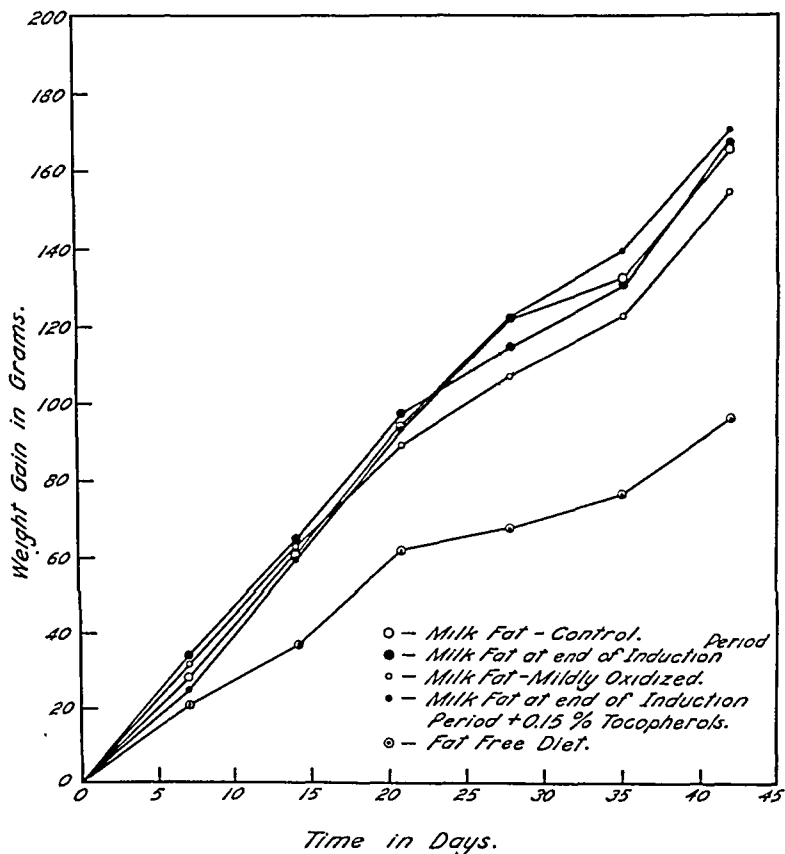


Fig. 5 The growth of young rats on diets containing milk fat in different stages of oxidation.

#### DISCUSSION OF RESULTS

The growth-retarding effects of solvents on fats to be used in experimental diets evidently result from a residue of a

deleterious component, where the fat has not interacted chemically with the solvent, and where the fat has not become oxidized. Reduction of the oxidative stability, short of actual oxidation, in these experiments, did not affect the growth of young rats when fed diets containing milk fat treated thusly. Also, mildly oxidized fat did not reduce growth to the same extent as the unpurified pentane.

Noteworthy is the significantly greater growth resulting from the diet containing the  $-53^{\circ}$  filtrate milk-fat fraction prepared with purified pentane. The animals on this diet were able also to use their feed more efficiently. It seems probable that a growth-promoting component from milk fat is concentrated in this fraction. Studies are under way to identify this possible component and to determine its mode of action.

The authors do not have any satisfactory explanation to account for the differences in weight gains as represented by the same diets in figures 2, 4 and 5.

#### SUMMARY AND CONCLUSIONS

1. Experiments have shown that pentane (Skelly-Solve A) contains an impurity which lowers the growth-promoting value of milk fat. The oxidative stability is also lowered.
2. Purification of the solvent by treating with fuming sulphuric acid removes this deleterious component.
3. Fat dissolved in purified pentane is not reduced in oxidative stability nor in growth-promoting value.
4. The  $-53^{\circ}$  filtrate milk-fat fraction prepared with purified pentane produced significantly greater growth in young rats than the original milk fat and also greater than the other milk fat fractions.
5. Young rats fed a diet containing  $-53^{\circ}$  filtrate milk-fat fraction used their feed more efficiently for growth than those fed diets containing the original milk fat and the other fractions.

## ACKNOWLEDGMENTS

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# AMINO ACID DEFICIENCIES OF RAW AND OVER-HEATED SOYBEAN OIL MEAL FOR CHICKS<sup>1</sup>

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Morgan ('31) found that the biological value for rats of cereal proteins and casein was decreased by dry heating at 140°C. for 30 minutes. Later it was shown (Greaves, Morgan and Loveen, '38) that lysine was very effective in correcting the amino acid deficiency of heated casein. Boiling or autoclaving of meat protein (Morgan and Kern, '34) and autoclaving of edestin (Waisman and Elvehjem, '38) for 5 hours at 120°C. reduced the biological value of these proteins for rats. A supplement of lysine to the autoclaved edestin gave better growth than did the unautoclaved material. Overheating of cereal and other proteins in the explosion process used in the preparation of many breakfast foods caused a marked decrease in protein nutritive value (Stewart, Hensley and Peters, '43; Mitchell, Hamilton and Beadles, '45).

The nutritive value of soybean oil meal was shown to be improved by proper heat treatment and impaired by autoclaving for too long a time or at too high a temperature (Parsons et al., '39; Bird and Burkhardt, '43; Evans and McGinnis, '46).

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Parsons et al. ('39) reported that lysine failed to improve the nutritive value of over-heated soybean oil meal. Evans and McGinnis ('46), using a diet containing 5% dried brewers' yeast and 5% gelatin in addition to the soybean oil meal, found that methionine added to the diet containing over-heated soybean oil meal (autoclaved for 30 minutes at 130°C.) gave a chick growth response almost equal to that obtained with optimally heated soybean oil meal plus methionine.

Clandinin et al. ('47) found that over-heated soybean oil meal, when incorporated in a practical soybean oil meal chick diet, was deficient in available methionine and lysine. Since in the earlier work (Evans and McGinnis, '46; Clandinin et al., '47) all of the dietary protein was not supplied by soybean oil meal, it was desirable to determine whether amino acids in soybean oil meal other than methionine and lysine are made unavailable to the chick by prolonged autoclaving.

#### EXPERIMENTAL

The raw soybean oil meal used in this study was a solvent-extracted product. The required amount for each diet was spread in enamel pans to a depth of about 1 inch and then autoclaved. As indicated in table 1, some samples were autoclaved for 30 minutes at 100°C., whereas, others were autoclaved for 60 minutes at 130°C. These treatments had previously been found (Evans and McGinnis, '46) to give a soybean oil meal with high and low nutritive value, respectively.

The percentage composition of the diets fed was as follows: Soybean oil meal 48.0, cerelese 43.9, mineral mixture<sup>3</sup> 5.0, soybean oil 2.5, fish oil (400 A.O.A.C. units D, 1000 U.S.P. units A/gm) 0.5, ethanol-soluble liver fraction 0.1. Vitamins were added in milligrams to each 100 gm of diet as follows: choline chloride 200, mixed tocopherol concentrate 10.0, p-aminobenzoic acid 10.0, niacin 3.0, 2-methyl 1, 4-naphtho-

<sup>3</sup> Composition of mineral mixture (grams): Ground limestone 1570, dicalcium phosphate 1740,  $K_2HPO_4$  840, NaCl 600,  $MgSO_4 \cdot 7 H_2O$  500,  $Fe (SO_4)_2 \cdot x H_2O$  55,  $MnSO_4 \cdot 4 H_2O$  29, KI 3.3,  $CuSO_4 \cdot 5 H_2O$  1.5, ZnCl<sub>2</sub> 1.0,  $CoCl_2 \cdot 6 H_2O$  0.2.

quinone 1.0, riboflavin 1.0, calcium pantothenate 1.0, thiamine 0.5, pyridoxine HCl 0.5, pteroylglutamic acid 0.05, biotin 0.01. The diets contained 22.4% protein, 0.26% methionine and 0.46% cystine. The methionine and cystine were determined by the differential oxidation procedure (Evans, '45). The only differences between the various diets were the different autoclaving treatments that the raw soybean oil meal received and the amino acid supplements which are described in table 1. The amount of cystine and methionine added to the diets was sufficient to meet the chick's requirement for these 2 amino acids, according to Grau and Almquist ('43).

Unsexed New Hampshire chicks were distributed at random into 30 groups of 10 chicks each. The chicks were wing banded and placed in electrically heated battery brooders. Each experimental diet and water were fed *ad libitum* to duplicate groups of chicks. The chicks were weighed individually and feed consumption determined at weekly intervals. The duration of the experiment was 4 weeks.

#### RESULTS

The results on chick growth and feed utilization are summarized in table 1. The unautoclaved raw soybean oil meal gave poor growth, and a supplement of cystine failed to appreciably improve the growth of chicks fed the raw soybean oil meal. Methionine gave a much greater growth response than cystine but failed to give maximum growth. A combination of methionine and cystine gave about the same growth as methionine alone.

Autoclaving the raw soybean oil meal for 30 minutes at 100°C. gave a pronounced growth response which was more than twice as great as that given by the supplement of methionine to the raw soybean oil meal. Growth of chicks fed diets containing soybean oil meal autoclaved for 30 minutes at 100°C. was not improved by supplements of either (1) methionine alone, (2) cystine alone, (3) methionine and cystine, or (4) methionine, cystine and lysine. These results together with the very satisfactory growth results at 4 weeks indicate

TABLE 1

*The influence of amino acid supplements on the nutritive value of soybean oil meal given different autoclaving treatments.*

AUTOCLAVING OF RAW SOYBEAN OIL MEAL		ADDITIONS TO DIET	AVERAGE WEIGHT AT 4 WEEKS		PROTEIN EFFICIENCY
<i>Time = min. Temp = °C.</i>		<i>gm/100 gm</i>	<i>gm</i>	<i>gm</i>	<i>(gain per gm protein)</i>
None	None	None	107	123 <sup>1</sup>	1.4
			138		
			131		
None	None	0.5 cystine	143	137	1.5
			207		
			184		
None	None	0.5 methionine	156	196	1.8
			186		
			212		
None	None	0.5 methionine 0.5 cystine	325	186	1.6
			287		
			319		
30	100	None	306	322	2.1
			287		
			319		
30	100	0.5 cystine	324	322	2.3
			284		
			284		
30	100	0.5 methionine	286	286	2.2
			288		
			304		
30	100	0.5 cystine 0.5 methionine	302	302	2.2
			300		
			295		
30	100	0.5 methionine 0.5 cystine 0.5 lysine	298	298	2.3
			301		
			161		
60	130	None	180	180	1.5
			198		
			164		
60	130	0.5 cystine	167	167	1.5
			169		
			166		
60	130	0.5 methionine	177	177	1.5
			188		
			213		
60	130	0.5 cystine 0.5 methionine	201	201	1.6
			186		
			157		
60	130	0.5 lysine	167	167	1.5
			176		
			290		
60	130	0.5 cystine 0.5 methionine 0.5 lysine	304	304	2.0
			319		

that 48% of soybean oil meal in the diet meets the chick's need for amino acids and also that autoclaving at 100°C. for 30 minutes did not destroy or render unavailable an appreciable amount of methionine, cystine or lysine.

In contrast to the excellent growth results obtained with the soybean oil meal autoclaved at 100°C. for 30 minutes, chicks grew poorly when fed soybean oil meal autoclaved for 60 minutes at 130°C. This treatment had previously been found to decrease the retention of methionine and cystine by chicks (Evans and McGinnis, '46). In the earlier work in which soybean oil meal supplied about 60% of the dietary protein, a supplement of methionine to soybean oil meal autoclaved 30 minutes at 130°C. gave maximum growth. The results in table 1 show that supplementary methionine, cystine and lysine when added singly to the diet containing the overcooked soybean oil meal failed to improve growth. A combination of methionine and cystine did not correct the heat damage to the soybean oil meal protein. On the other hand, a combination of methionine, cystine and lysine when added to the diet containing overcooked soybean oil meal corrected the amino acid deficiencies of the heat-damaged soybean oil meal. Since a combination of cystine and lysine or of methionine and lysine was not used, it cannot be determined from the data whether methionine or cystine was destroyed or made unavailable by autoclaving soybean oil meal at 130°C. for 60 minutes.

#### DISCUSSION

The greater growth response obtained in this work as compared to that obtained earlier (Evans and McGinnis, '46) by autoclaving for 30 minutes at 100°C. is probably explained by the difference in the amount of dietary protein supplied by soybean oil meal. The diets used in the work reported in this paper contained all of the protein from soybean oil meal, whereas, the diets used in the earlier work (Evans and McGinnis, '46) had about 40% of the protein supplied by dried brewers' yeast and gelatin. Both of these materials are poor sources of methionine and cystine. Under the conditions of the

experiment reported in this paper, methionine was not a growth-limiting deficiency in the diets containing soybean oil meal autoclaved for 30 minutes at 100°C. On the other hand, according to Grau and Almquist ('43), the 0.26% of methionine and 0.46% cystine supplied by 48% of soybean oil meal would not be sufficient to meet the chick's requirement for these amino acids. Grau and Almquist ('43) reported that the chick needs about 0.55% methionine and a total of 1.0-1.1% of methionine and cystine for maximum growth.

The effect of methionine in correcting the heat damage to soybean oil meal caused by autoclaving for 30 minutes at 130°C. in the earlier work (Evans and McGinnis, '46) was probably due to the lysine supplied by the dried brewers' yeast and gelatin and to less destruction of lysine. The failure of lysine to correct the heat damage to soybean oil meal reported by Parsons et al. ('39) may have been due to a critical deficiency of methionine.

The decreased liberation of nitrogen from soybean oil meal by *in vitro* enzymatic digestion with pepsin, trypsin, and erepsin caused by overcooking (Evans, '46) suggests that the biological value might be impaired by rendering of the proteins indigestible. The data obtained with the chick by supplementing the heat-damaged soybean oil meal with methionine, cystine and lysine indicate that these particular amino acids rather than the entire proteins were affected by overcooking under the experimental conditions.

#### SUMMARY

Data obtained with chicks using a diet in which soybean oil meal supplied all of the protein permit the following conclusions regarding the effect of autoclaving on the biological value of soybean oil meal proteins:

1. Chick growth was not improved by supplementing a diet containing raw soybean oil meal with cystine. The addition of methionine to this diet gave a growth response that was not maximum.

2. Autoclaving raw soybean oil meal at 100°C. for 30 minutes gave a marked increase in its nutritive value as shown by chick growth. Neither methionine nor cystine nor a combination of these 2 amino acids improved chick growth when added to the diet containing soybean oil meal autoclaved at 100°C. for 30 minutes. Furthermore, a combination of cystine, methionine, and lysine failed to improve growth.

3. The nutritive value of soybean oil meal was decreased by autoclaving at 130°C. for 60 minutes. The heat damage to the soybean proteins caused by this treatment was not corrected by the addition of methionine, cystine, or lysine. A combination of these 3 amino acids corrected the heat damage caused by this autoclaving treatment.

4. The growth response given by autoclaving raw soybean oil meal at 100°C. for 30 minutes was more than twice as great as the response given by a supplement of methionine. This indicates that autoclaving raw soybean oil meal improves the nutritive value by affecting the availability of nutrients other than cystine or methionine.

5. The methionine requirement of the chick appeared to be not more than 0.26% when the diet contained 0.46% cystine. It may be possible that New Hampshire chicks require less methionine than White Leghorns.

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# RUMEN DIGESTION STUDIES

## I. A METHOD OF INVESTIGATING CHEMICAL CHANGES IN THE RUMEN<sup>1</sup>

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A universal practice among investigators of ruminant nutrition is one of frequent reference to the importance of the rumen in accomplishing the disintegration of plant materials. Yet, amazingly little is known of the nature and extent of such disintegration, of the responsible organisms, or of the relation of rumen action to subsequent action in other divisions of the digestive tract. Consequently, a comprehensive concept of rumen digestion has not been possible. Progress in the elucidation of the chemical changes taking place in the rumen has been slow due to a lack of appropriate experimental techniques. In early studies with fistulated animals results were interpreted in terms of the percentage composition of the rumen contents as compared with the hay fed (Krzywanek and Quittek, '36) or by comparing the composition of the rumen contents as digestion progressed (Silver, '35). Burroughs, Gerlaugh, Silver and Schalk ('46) reported a chemical method for identifying individual feeds within the rumen of cattle receiving a mixed ration containing corn, hay and a protein supplement. This method was based upon a mathematical treatment of the principle nutrients contained in the ingested feeds and the nutrients present in the rumen ingesta.

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Studies of this nature only yield data of relative value and give little indication of the actual changes. Hale, Duncan and Huffman ('40), however, pointed out the application of lignin ratios to investigations of rumen digestion, and McAnally ('42) reported on the value of suspending silk sacs containing ground straw in the rumen. These methods seem to circumvent many of the complications introduced by a moving system and promise to yield information of a fundamental nature.

In the present paper a critical appraisal of the lignin ratio method is made and a technique for measuring the rate of rumen digestion is presented. In a previous study (Hale, Duncan and Huffman, '40) lignin was assumed to be indigestible in the rumen, whereas the present communication demonstrates a method of measuring the digestibility of lignin in the rumen.

#### METHODS

*Chemical procedures.* Lignin, cellulose and other carbohydrates were determined by the procedures of Crampton and Maynard ('38) and all the other nutrient values were obtained by the A.O.A.C. methods ('40).

*Measurement of concentration by means of lignin ratios.* In a preceding paper (Hale, Duncan and Huffman, '40) lignin ratios were used to calculate rumen digestion coefficients. This method was based upon the assumption that lignin was not digested to any appreciable extent in the rumen. An assumption of this character permits a measurement of the concentration of the rumen contents by comparing the per cent of lignin in the rumen with that determined in the ingested hay. The method of calculating the rumen coefficients is represented in the following formula:

$$(1) \quad 100 - \left( \frac{\text{Per cent lignin in hay}}{\text{Per cent nutrient in hay}} \right) \times \left( \frac{\text{Per cent nutrient in rumen}}{\text{Per cent lignin in rumen}} \right) \times 100 =$$

Digestion coefficient of nutrient

The validity of this method is dependent upon the degree to which lignin is attacked in the rumen. Evidence of the indigestibility of lignin was obtained by application of the

method to rumen materials (Hale, Duncan and Huffman, '40). Any digestion of lignin would have resulted in low digestion coefficients, yet calculated rumen coefficients were high and approached or equaled fecal coefficients. Data of this nature suggest that lignin does not undergo any appreciable digestion in the rumen; nevertheless, more specific information regarding this point is necessary. Digestion trials reported by Ellis, Matrone and Maynard ('46) indicate that lignin is not digested by the cow, sheep and rabbit.

To fill the need for a measurement of the digestibility of lignin in the rumen, thereby enabling the investigator to correct rumen digestion coefficients for any discrepancies arising from lignin digestion, the following formula may be used:

$$(2)^2 \quad \frac{\left( \frac{\text{Pounds of crude fiber digested}}{\text{Pounds of lignin in hay}} + \frac{\text{Pounds of N.F.E. digested}}{\text{Pounds of lignin in hay}} \right) - \left( \frac{\text{Pounds of cellulose digested}}{\text{Pounds of lignin in hay}} + \frac{\text{Pounds of other CHO digested}}{\text{Pounds of lignin in hay}} \right)}{\text{Digestion coefficient of lignin}} \times 100 =$$

This formula is possible since the sum of crude fiber and nitrogen-free extract is identical to the sum of cellulose, other carbohydrates and lignin. Thus any difference in the digestibility of the 2 fractions represented in the formula would be due to the digestibility of lignin. The digestion coefficients derived from formula (1) were obtained originally by assuming lignin to be indigestible. Although any digestion of lignin would result in calculated coefficients lower than the real coefficients, the error would be of the same magnitude for both of the involved fractions. The accuracy of the calculation would not be appreciably impaired because the difference between these 2 fractions would remain essentially unaltered.

An idea of the applicability of formula (2) may be obtained by applying it to fecal material of known digestibility coefficients. By using the data in columns 1 and 7 of table 2 and columns 3 and 8 of table 3 in the accompanying paper Hale, Duncan and Huffman ('47) found the calculated lignin digestion coefficients to be 31.3 and 21.9% as compared with the respective actual values of 32.7 and 21.5% (table 3). These

<sup>2</sup> Pounds of a nutrient refers to the amount per 100 pounds of hay.

values appear to be within the limits of the experimental error encountered in digestion trials.

Corrections for rumen digestion coefficients obtained by formula (1) can readily be made if an appreciable digestion of lignin is encountered in the rumen. In making the calculations, the percentage of indigestible lignin in the hay is used rather than the original lignin content of that hay. For example, if the hay contained 15.0% lignin and if 4% of the lignin was digested in the rumen, then  $0.15 - (0.15 \times 0.04) \times 100$  or 14.4 would replace the 15.0% value in the formula for calculating the rumen coefficient. Thus, a formula free of any discrepancies introduced by the possible disintegration of lignin in the rumen is obtained with which rumen digestion coefficients can be calculated:

$$(3) \quad 100 - \left( \frac{\text{Per cent indigestible lignin in hay}^4}{\text{Per cent nutrient in hay}} \times \frac{\text{Per cent nutrient in rumen}}{\text{Per cent lignin in rumen}} \right) \times 100 =$$

Digestion coefficient of nutrient

In table 3 in the accompanying paper (Hale, Duncan and Huffman, '47) both the original and corrected coefficients of rumen digestion are presented.

*Limitations of the lignin ratio method.* The lignin ratio method is not adapted to the study of rations containing concentrates and it gives significant values for roughages only after rumen digestion has reached a maximum.

As concentrates are deficient in lignin a suitable lignin value cannot be obtained for measuring their concentration. In a mixed ration differences in the rate of passage of concentrates and fibrous materials would invalidate the calculations. Investigations of the rumen disintegration of concentrates and mixed rations might better be carried out by means of the silk-sac method described by McAnally ('42). Results obtained by this method are limited to comparative interpretations alone since concentrates would be retained in the rumen beyond the period of normal passage and, as McAnally's data for straw suggest, a retarded digestion may occur (3 days required for 40% digestion). The simplicity of this method,

\* This term refers to indigestibility in the rumen.

however, and the large number of samples that may be studied recommend its application in comparative and preliminary investigations.

The limitation of the lignin ratio method for any measurement other than that of the maximum concentration in the rumen can readily be noted. When an animal consumes the feed placed before it, the freshly ingested material is mixed with the highly concentrated rumen contents of the previous digestion period. This means that a sample collected from the rumen immediately after feeding would indicate, on the basis of lignin ratios which measure concentration, that considerable disintegration had occurred while in reality only a mechanical mixing of the hay and the previously concentrated rumen contents had taken place. If simple lignin ratios are used to calculate the digestibility of dry matter in the rumen at zero hours after feeding on the basis of the data in table 2 in the accompanying paper (Hale, Duncan and Huffman, '47), a value of 38.3% would be obtained. This value represents mechanical concentration only and indicates the magnitude of the error resulting from such concentration. The results obtained by Rathnow ('38) further demonstrate this limitation. He used the simple ratio method, employing iron rather than lignin, for studies of the rate of rumen digestion and obtained values of 32.1, 48.5 and 58.8% for the digestibility of dry matter at 3, 6 and 9 hours, respectively, after ingestion. These values, particularly those for 3 and 6 hours, are unduly high and are of the magnitude that might be expected from mixing the ingesta with the concentrated rumen contents.

As lignin ratios do not differentiate between mechanical concentration and concentration resulting from current disintegrative activity, they are limited to the determination of the maximum concentration occurring in the rumen. For this purpose the method would seem well adapted and subject only to those errors innate in ordinary digestion trials.

*Measurement of the passage of nutrients from the rumen or a method for studying the rate of rumen digestion.* A moving system such as that encountered in the study of rumen diges-

tion presents complications which must be thoroughly understood before undertaking an investigation of the rates of chemical changes. In the course of a 12-hour digestion period between feedings passage of nutrients from the rumen is effected by 2 distinct processes. These are (a) passage of the concentrated rumen contents remaining in the rumen from previous digestion periods,<sup>5</sup> and (b) separation of nutrients from the plant material recently ingested and subsequently washed from the rumen. Thus many nutrients pass from the rumen in both an indigestible and a digestible form. Any measure of rumen digestion other than that of maximum digestion must, therefore, differentiate between these 2 types of passage.

A clear distinction between these 2 types of passage is dependent upon the degree of completion of the digestive processes within the 12-hour period following feeding. If rumen digestion is complete at 12 hours after feeding, the rumen contents present at that time would not undergo any further change and all digestion taking place during the next 12 hours would represent digestion of the newly ingested material. It is important, therefore, to examine evidence relating to the completeness of rumen action in the course of the normal 12-hour feeding interval. Hale, Duncan and Huffman ('40) removed rumen contents from cows at 14 and 24 hours after feeding. Rumen digestion coefficients as measured by lignin ratios were essentially unchanged, although half of the dry matter present in the rumen at 14 hours had passed from the rumen during the succeeding 10 hours. Excluding ether extract, which is complicated by a synthesis of fat, the highest value for rumen digestion observed for the 14-24-hour period was only 1.8%. This value is within the limits of experimental error. It might be suggested that even if further digestion had occurred a proportionate removal of lignin might obscure this digestion. Calculations of lignin digestion

<sup>5</sup> This refers to any concentration of the rumen contents irrespective of the digestion period and also includes the material reaching a maximum concentration in the current digestion period.

using formula (2) eliminate this possibility as zero values for lignin digestion were obtained at both 14 and 24 hours. A comparison of rumen concentration at 12 hours with fecal concentration gives further evidence of completed digestion. Under conditions at the Michigan Station average rumen coefficients at 12 hours for the dry matter in different lots of roughage varied from 45.7 to 48.7% while fecal coefficients for the same lots of roughage varied from 46.7 to 59.8%. Rumen concentration at 12 hours after feeding is thus seen to approach and, in some instances, to equal fecal concentration. The uniformity of the rumen values in contrast with the variable fecal values further suggests a "ceiling" on rumen digestion corresponding to the lowest fecal concentrations observed in digestion trials. All rumen digestion coefficients for 12 hours have approximated the level of such a hypothetical "ceiling" and suggest that the maximum limits of rumen digestion are normally reached within the 12-hour digestion period. The problem of a "ceiling" for rumen digestion is discussed further in the accompanying paper (Hale, Duncan and Huffman, '47). Miscellaneous observations of rumen physiology also suggest that the peak of digestion is reached within 12 hours after feeding. The rumen pH values begin to drop and reach a maximum acidity approximately 6 hours after feeding, after which time the values gradually increase to the prefeeding level (Monroe and Perkins, '39; Hale, Duncan and Huffman, '40; Myburgh and Quin, '43).

The microbiological studies of Bortree ('46) on the changes in rumen microflora from feeding might be applicable here. The conclusion that rumen digestion comes to an end prior to 12 hours after feeding would appear tenable. We may, therefore, proceed to a measurement of the different types of passage and to a calculation of rumen coefficients.

In general a digestion coefficient is calculated by dividing the amount of a nutrient digested by the amount of the same nutrient in the feed and multiplying the result by 100. The amount of a particular nutrient digested in the rumen, and

hence the rumen digestion coefficient can be determined on the basis of the following relationship:

$$(a) \text{ Amount of nutrient digested in rumen } = \left( \text{Total passage of the nutrient from the rumen} - \text{Passage of nutrient in the undigested form} \right)$$

Measurement of the total passage of dry matter, or of a particular nutrient, from the rumen can readily be accomplished by removing and weighing the entire contents of the rumen at the time of feeding and again at the end of the digestion period under investigation. The total nutrients in the rumen after feeding are represented by the sum of the rumen contents at feeding and the ingested hay. The amount of nutrients present at the end of the specified digestion period is subtracted from this total to give the total passage of nutrients from the rumen. As the total passage of nutrients from the rumen can thus be measured, the problem becomes one of measuring the passage of the concentrated rumen contents remaining from the previous digestion periods,<sup>6</sup> i.e., the passage of undigested nutrients.

Lignin values would seem readily adaptable for such a measurement. The percentage of lignin in the rumen contents at 12 hours also represents the percentage of lignin in the maximally concentrated rumen contents since digestion reaches a maximal level within the 12-hour period. The total amount of lignin passing from the rumen can be measured as above. If lignin leaves the rumen exclusively as a component of the maximally concentrated contents we may then calculate the total passage of these concentrated rumen contents as follows:

$$(b) \frac{\text{Amount of lignin leaving rumen}}{\text{Per cent lignin in rumen at 12 hours}} = \frac{\text{Total amount of concentrated contents passing from rumen}}{\text{Total amount of concentrated contents passing from rumen}}$$

The amount of undigested nutrients passing from the rumen during the digestion period under investigation can be determined from formula (b).

A question might be raised as to whether or not lignin leaves the rumen exclusively as a component of the maximally

<sup>6</sup> Here again, this includes any concentrated rumen contents passing from the rumen, irrespective of the time that it was consumed.



concentrated rumen contents. As only small amounts of lignin are normally digested in the rumen (Hale, Duncan and Huffman, '47) and since such digestion can readily be corrected for by the use of formula (2), the possibility of lignin passing in a digested state does not present a problem. The only remaining path of passage would be as a component of plant fragments not yet subjected to the concentrating action of the rumen. The selective passage of highly comminuted material from the rumen as noted by Schalk and Amadon ('28), however, suggests that the passage of plant fragments not yet subjected to the concentrating action of the rumen would not be significant.

Formula (b) can be extended for a measurement of the passage of any particular nutrient in the undigested state since both the amount and composition of the concentrated rumen contents passing from the rumen can be determined. The calculation is represented as follows:

$$(c) \left( \frac{\text{Amount of lignin leaving rumen}}{\text{Per cent lignin in rumen at 12 hours}} \times \text{Per cent nutrient in rumen at 12 hours} \right) = \text{Amount of nutrient in undigested state}$$

The formula for measuring rumen digestion coefficients may now be expressed in the following completed form:

$$(4) \frac{\left( \frac{\text{Total amount of nutrient leaving rumen}}{\text{Per cent lignin in rumen at 12 hours}} - \frac{\text{Amount of lignin leaving rumen}}{\text{Per cent lignin in rumen at 12 hours}} \right) \times \text{Per cent nutrient in rumen at 12 hours}}{\text{Amount of nutrient in hay}} \times 100 = \text{Digestion coefficient of nutrient}$$

This formula may be applied to studies of the extent of rumen digestion of any particular nutrient at any given period after feeding, thereby permitting an investigation of the rate of rumen digestion. The method proposed for calculating digestion coefficients for dry matter and protein at 6 hours after feeding is illustrated by the following examples.

#### Data.

1. Cow received 11.3 pounds of hay containing 15.2% lignin, 100% dry matter and 15.5% protein.

2. Rumen contents at feeding time (i.e., 12 hours after previous feeding) contained 25.2 pounds of dry matter, 28.9% lignin and 12.5% protein.

3. Rumen contents 6 hours after feeding contained 30.2 pounds of dry matter, 26.2% lignin and 12.8% protein.

The total passage of all nutrients (i.e., dry matter) and of lignin is needed to complete the values required in formula (4).

(a) Calculation of digestibility of dry matter:

$$\begin{aligned} 11.3 + 25.2 &= 36.5 \text{ lbs. of dry matter in rumen after feeding} \\ 36.5 - 30.2 &= 6.3 \text{ lbs. of dry matter which left rumen in 6 hours} \\ 25.2 \times 0.289 &= 7.3 \text{ lbs. of lignin in rumen before feeding} \\ 11.3 \times 0.152 &= 1.7 \text{ lbs. of lignin in hay} \end{aligned}$$

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$$\text{Total} = 9.0 \text{ lbs. of lignin in rumen after feeding}$$

$$30.2 \times 0.262 = 7.9 \text{ lbs. of lignin in rumen at 6 hours}$$


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$$\text{Difference} = 1.1 \text{ lbs. of lignin which left rumen in 6 hours}$$

Substitution in formula (4) gives

$$6.3 - \left( \frac{1.1 \times 1.00}{0.289} \right) \times 100 = 22.1\% \text{ dry matter digested.}$$

(b) Calculation of digestibility of protein:

$$\begin{aligned} 25.2 \times 0.125 &= 3.2 \text{ lbs. protein in rumen before feeding} \\ 11.3 \times 0.155 &= 1.8 \text{ lbs. of protein in hay} \end{aligned}$$

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$$\text{Total} = 5.0 \text{ lbs. of protein in rumen after feeding}$$

$$30.2 \times 0.128 = 3.9 \text{ lbs. of protein in rumen at 6 hours}$$


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$$\text{Difference} = 1.1 \text{ lbs. of protein which left rumen in 6 hours}$$

Substitution in formula (4) gives

$$1.1 - \left( \frac{1.1 \times 0.125}{0.289} \right) \times 100 = 33.3\% \text{ protein digested.}$$

Results obtained by applying the above method to studies of the rate of rumen digestion are presented in the accompanying paper (Hale, Duncan and Huffman, '47).

#### DISCUSSION

*Recapitulation of methods.* The theoretical and experimental basis for the calculation of rumen digestion coefficients

has been adequately discussed in the presentation of the methods and need not be commented on further.

In investigations of the fate of roughage in the rumen the investigator can employ either of 2 methods. In any study where a measure of the maximum digestion alone is desired formula (3) should be employed because of its greater simplicity and because only 1 removal of rumen contents is required. For investigations of the rate of rumen digestion formula (3) is inadequate and the investigator must apply formula (4) to obtain significant values. In this instance rumen contents are removed at the beginning of the digestion period under consideration and again at 12 hours after feeding. Since maximum digestion is achieved within 12 hours, both methods may be applied at or after that time with identical results.

Lignin values form the basis of both of these methods but are used in 2 distinct ways. In the first instance they are used to indicate the ratio between the lignin of the hay and that of the rumen contents thereby giving a measure of the concentration of the hay in the rumen. In the second instance lignin values are not used to measure concentration but rather to measure the amount of nutrients passing from the rumen in the digested and undigested form. The actual amounts are then used to calculate digestion coefficients. A method of measuring the rumen digestion of lignin is presented in formula (2) so that any discrepancies resulting from the digestion of lignin in the rumen may be eliminated.

These methods, while seemingly well adapted to studies with roughages, are not suitable to studies of mixed rations since concentrates are low in lignin and pass from the rumen more rapidly than roughages. It is suggested that rations containing concentrates may best be studied by use of the silk-sac method employed by McAnally ('42). The limitations of this method have been noted.

Problems of the chemical changes occurring in the rumen readily yield themselves to investigation by a combination of the methods described herein.

*What is measured?* Some question might arise as to whether these methods measure true digestion, i.e., the effective solution of nutrients, or a mere mechanical passage of plant particles which escaped the digestive processes. In view of the selective passage of highly comminuted material from the rumen (Schalk and Amadon, '28) and the tendency toward stagnation in the rumen, it is unlikely that any appreciable passage of plant particles not yet thoroughly subjected to the disintegrative action of the rumen would take place. This means that passage from the rumen is almost wholly in the form of either maximally concentrated rumen contents or of soluble nutrients. As the method presented herein differentiates between these 2 types of passage, it may be considered to measure an actual solution of nutrients.

Values for rumen digestion coefficients obtained by the methods presented are neither unduly high nor low (Hale, Duncan and Huffman, '47). They are well distributed over the entire range of possible values with nutrients of high digestibility approaching the upper limits and those of low digestibility nearing the lower. This condition is not likely to be obtained if any appreciable amount of plant material passed from the rumen before reaching the maximum state of digestion. Extremes observed were the zero digestion coefficient for crude fiber at 6 hours after feeding and the 65.2 and 83.0% digestion at 12 hours for nitrogen-free extract and other carbohydrates, respectively. The last 2 values represented 100.2 and 101.6% of the fecal concentration of these highly digestible nutrients, an unusually precise coincidence.

#### SUMMARY

Methods for calculating the maximum digestion in the rumen on the basis of lignin values have been extended to include techniques for measuring the rate of rumen digestion and the digestibility of lignin in the rumen.

Principles forming the basis for rumen digestion calculations receive special attention.

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# RUMEN DIGESTION STUDIES

## II. STUDIES IN THE CHEMISTRY OF RUMEN DIGESTION<sup>1</sup>

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By applying new techniques to studies of rumen digestion of alfalfa hay in the bovine, Hale, Duncan and Huffman ('40) first demonstrated the magnitude of the chemical changes taking place in the rumen. Since that time the method of investigation has been extended to permit a study of the rate, as well as the magnitude, of these chemical changes (Hale, Duncan and Huffman, '47) and the results have been compared quantitatively with digestion in other portions of the digestive tract. The microbiological aspects of the chemical changes have been considered, with special emphasis on fat synthesis. The results obtained by applying these methods to studies on the rate of rumen digestion are presented in this paper.

### METHODS

Rumen digestion coefficients were measured by the lignin-value method of Hale, Duncan and Huffman ('47). In all trials with the 2 rumen fistula cows the entire contents of the rumen were removed, weighed, mixed, sampled and returned immediately to the rumen after a representative sample of 7-10 pounds had been taken. Enough alcohol was added to give approximately a 50% concentration. The samples were stored

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in the cold and subsequently dried in a current of warm air at 24°C. Samples were removed from cow R-4 at 6 and 12 hours and from animal R-1 at 14 and 24 hours after feeding. A period of from 1 to 2 weeks was allowed between such removals of rumen content to insure complete resumption of the normal function of the rumen.

In special studies of fat synthesis 3 cows were fed basic beet pulp rations low in fat. The specific rations are given in table 5. Representative samples were taken from the rumen of cow R-1 which was fed 2 different beet pulp rations. The 2 cows, C-167 and D-11, were slaughtered and representative samples of the rumen contents were taken immediately. These samples were taken to the laboratory and dried at once on the steam bath. Since lignin values are not applicable for measuring the concentration of the beet pulp rations in the rumen, the concentration was assumed to be 90% of the fecal concentration, a value suggested by studies of the rumen concentration of alfalfa. Morrison ('45) gives 71.8% for the fecal digestion of dried beet pulp. Ninety per cent of this value gives 64.6 as the maximum concentration likely to occur in the rumen. Under the conditions of this experiment, the theoretical increase of fat in the rumen can be calculated as follows when the beet pulp ration comprises 0.43% fat and the rumen contents actually contain 2.71% fat 8 hours after feeding (table 5):

$$\begin{aligned} (.0043 \times .646) \times 100 &= 0.28\% \text{ increase of fat due to concentration} \\ 0.43 + 0.28 &= 0.71\% \text{ fat after concentration occurred (theoretical)} \\ \left( \frac{2.71 - 0.71}{0.71} \right) \times 100 &= 281.7\% \text{ actual increase of fat in rumen} \end{aligned}$$

Differences in the gross and net increases observed in table 5 emphasize the importance of eliminating increases due to the concentration of digestion when evaluating rumen synthesis in order to make the final test for fat synthesis more critical.

Lignin, cellulose and other carbohydrates were determined by the procedures of Crampton and Maynard ('38), fatty acids by the method of Horwitt, Cowgill and Mendel ('36), and all other nutrient values by the A.O.A.C. methods ('40).



## RESULTS

Data relating to the removal of rumen contents are presented in table 1, and the percentage composition of the alfalfa hay and rumen contents is shown in table 2. Rumen digestion coefficients calculated at various periods subsequent to feeding are presented in table 3. Values for 12 hours or more after feeding were obtained by using formula (1) and those for less than 12 hours by formula (4) of Hale, Duncan and Huffman ('47). Fecal digestion was determined by the usual digestion trials. In all instances coefficients for the rumen digestion of lignin were calculated by using formula (2) of the above

TABLE 1

*The amount and percentage of dry matter in the rumen.*

HOURS AFTER FEEDING	DRY MATTER FED	TOTAL CONTENT OF RUMEN	DRY MATTER IN RUMEN	
	lbs.	lbs.	%	lbs.
		Cow no. R-4		
6	11.3	185	16.3	30.2
12	11.3	177	14.6	25.8
6	11.3	185	16.2	30.0
12	11.3	174	14.2	24.7
		Cow no. R-1		
14	8.8	132	14.2	18.7
24	8.8	65	14.5	9.4

TABLE 2

*Percentage composition of alfalfa hay and rumen contents on the dry basis.*

NUTRIENT	COW NO. R-4			COW NO. R-1			AV. OF 8 TRIALS	
	Alfalfa hay	Rumen		Alfalfa hay	Rumen		Alfalfa hay	Rumen
		6 hr.	12 hr.		14 hr.	24 hr.		
Protein	15.5	12.8	12.5	17.0	11.8	11.0	16.6	13.0
Ether extract	1.6	2.5	2.1	1.3	1.9	1.5	2.0	2.5
Crude fiber	31.4	43.6	44.8	38.1	52.0	51.5	33.3	47.0
N-F extract	45.1	31.9	31.5	36.9	24.7	24.6	41.1	27.7
Cellulose	34.7	37.0	36.7	31.3	37.8	34.6	33.6	36.8
Other CHO	26.6	12.3	10.7	26.1	4.0	8.5	24.9	8.2
Lignin	15.2	26.2	28.9	17.5	34.8	33.0	16.1	30.2
Dry matter, lbs.	11.3	30.2	25.2	8.8	18.7	9.4		

authors. Rumen coefficients corrected for lignin digestion are given in column 7 of table 3 and were obtained by using formula (3) of Hale, Duncan and Huffman ('47). The rumen coefficients at 12, 14 and 24 hours were not in need of correction as lignin was not observed to be digested in those trials. At 6 hours, 3.7% lignin was observed to be digested but since the value at 12 hours for the same lot of hay and the same animal was —0.4% the first value was considered to be largely an experimental error and corrections were not made. The average rumen coefficients for 8 trials at 12 to 14 hours after

TABLE 3  
*Rumen and fecal digestion coefficients.*

NUTRIENT	COW NO. R-4			COW NO. R-1		AV. OF 8 TRIALS		
	Rumen		Fecal	Rumen		Rumen		Fecal
	6 hr.	12 hr.		14 hr.	24 hr.	12-14 hr.	Cor- rected <sup>1</sup>	
Dry matter	22.1	47.8	59.8	50.0	46.6	47.1	48.4	57.0
Protein	33.3	61.1	69.7	66.7	66.7	58.6	59.6	69.1
Ether extract	—72.2	33.3	43.2	26.3	38.6	32.5	35.4	19.3
Crude fiber	—0.3	25.7	49.6	32.4	29.4	25.5	27.2	46.9
N-F extract	45.1	62.7	68.5	65.6	62.5	64.6	65.2	65.1
Cellulose	12.8	43.6	63.1	39.3	42.9	42.1	43.4	55.0
Other CHO	53.3	80.0	74.7	91.3	82.6	81.8	83.0	81.7
Lignin	3.7	—0.4	32.7	0.0	0.0	3.1	3.1	21.5

<sup>1</sup> Corrected for lignin digestion.

feeding include data previously reported by Hale, Duncan and Huffman ('40). This number of trials should establish the maximum digestion of alfalfa hay in the rumen with some degree of certainty. A more graphic presentation of the rate of rumen digestion and of digestion in the remainder of the digestive tract is given in table 4. Data for cow R-4 were used to calculate digestion coefficients for the first and second 6-hour periods and data for cow R-1 to calculate the coefficients for the second 12-hour periods. Average rumen and fecal coefficients for the 8 trials were used in computing digestion coefficients after passage of material from the rumen.

Dry matter was digested at an even rate throughout the 12-hour digestion period with an average digestion of 48.4%

obtained at 12-14 hours. Protein, nitrogen-free extract and other carbohydrates were very rapidly removed during the first 6 hours. During the second 6 hours these nutrients were removed at approximately the same rate as the other nutrients. Average coefficients at the 12-14-hour period were 59.6, 65.2 and 83.0%, respectively. Crude fiber was not digested during the first 6 hours but the average coefficient at 12-14 hours was 27.2%. Apparent lignin digestion was only 3.1% at the 12-14-hour period (table 3). Cellulose was digested largely during the second 6-hour period when 30.8% was digested as contrasted with 12.8% digested during the first 6 hours (table 4).

TABLE 4

*The disintegration of alfalfa hay in the ruminant digestive tract.*

NUTRIENT	RUMEN			DIGESTION AFTER LEAVING RUMEN <sup>2</sup>	RUMEN DIGESTION AS PER CENT OF FECAL DIGESTION <sup>4</sup>
	1st 6 hr.	2nd 6 hr. <sup>1</sup>	2nd 12 hr. <sup>2</sup>		
	%	%	%	%	%
Dry matter	22.1	25.7	-3.4	8.6	84.9
Protein	33.3	27.8	0.0	9.5	86.3
Ether extract	-72.2	105.5	12.3	-16.1	
Crude fiber	-0.3	26.0	-3.0	19.7	58.0
N-F extract	45.1	17.6	-3.1	-0.1	100.2
Cellulose	12.8	30.8	3.6	11.6	78.9
Other CHO	53.3	26.7	-8.7	-1.3	101.6
Lignin	3.7	-4.1	0.0	18.4	14.4

<sup>1</sup> Difference between columns 1 and 2 in table 3.

<sup>2</sup> Difference between columns 4 and 5 in table 3.

<sup>3</sup> Difference between columns 7 and 8 in table 3.

<sup>4</sup> Obtained from columns 7 and 8 in table 3.

When removal of the rumen contents was delayed until 24 hours after feeding, the highest increase in digestion, excluding ether extract which is complicated by fat synthesis, was only 3.6% (table 4). This slight digestion of cellulose during the second 12 hours is in marked contrast to the great activity of the first 12 hours. The digestion of lignin after 24 hours in the rumen was observed to be zero in this instance.

After the plant fragments passed from the rumen an average of 8.6% of the dry matter was digested (table 4). Nitro-

gen-free extract and other carbohydrates were not further digested after leaving the rumen. Of the other nutrients protein averaged 9.5, lignin 18.4, cellulose 11.6, and crude fiber 19.7% digestibility in the lower digestive tract. The average lignin digestion of 18.4% is in marked contrast to the average rumen value of 3.1%. Lignin was the only nutrient digested to a greater extent after passing from the rumen than while in the rumen.

TABLE 5

*Increase in the ether extract content of the rumen over that in the ration.*

COW NO.	RATION	TIME AFTER FEEDING	ETHER EXTRACT IN RATION	ETHER EXTRACT IN RUMEN	GROSS INCREASE	NET INCREASE <sup>1</sup>
		hrs.	%	%	%	%
R-4	25 lbs. alfalfa	6	1.62	2.46	51.7	54.9
R-4	25 lbs. alfalfa	12	1.62	2.14	32.1	-28.6
R-1	30 lbs. alfalfa	14	0.50	1.10	120.0	26.3 <sup>2</sup>
R-1	20 lbs. alfalfa	14	0.69	0.83	20.6	-33.2 <sup>2</sup>
R-1	Beet pulp + C <sup>3</sup>	10	0.25	1.10	340.0	169.0 <sup>2</sup>
R-1	Beet pulp + M + S <sup>4</sup>	8	0.43	0.66	53.5	-7.0
C-167	Beet pulp + M + S <sup>4</sup>	8	0.43	1.39	223.3	95.8
D-11	Beet pulp + M + S <sup>4</sup>	8	0.43	2.71	530.2	281.7

<sup>1</sup> After increase due to concentration of digestion was eliminated.

<sup>2</sup> Fatty acid values used. All others are ether extract values.

<sup>3</sup> 12 lbs. beet pulp plus 0.25 lbs. casein.

<sup>4</sup> 10 lbs. beet pulp plus 5 lbs. molasses plus 2 lbs. starch.

When ether extract values were used with an alfalfa hay ration, synthesis was observed at the height of digestion only, i.e., 6 hours after feeding when an increase of 54.9% was noted. A summary of the data on fat synthesis is presented in table 5. During the second 6 hours there was a rapid dissipation of the accumulated fat which carried over into the second 12-hour period (table 4). When fatty acid values were used a synthesis was observed at 14 hours after feeding on a 30-pound hay ration but when the ration was reduced to 20 pounds per day a negative digestion coefficient of 33.2% was noted. Net increases in fat on the beet pulp ration varied from -7.0 to 281.7%. These variations occurred independently of the added

supplements. The highest value, 281.7%, was obtained from ether extract determinations. A high value of 169.0% was observed when fatty acid values were used.

#### DISCUSSION

Although many details of the basic chemical changes occurring in the rumen are as yet missing and stand in need of further investigation, sufficient data have been accumulated to permit a consideration of some of the salient features.

Dry matter was digested at an even rate throughout the 12-hour digestion period. During the first 6 hours the predominant phenomenon was the rapid disappearance of the more soluble nutrients, proteins and carbohydrates, from the rumen. Cellulose was only slightly digested and lignin and crude fiber escaped disintegration. The slow rate of digestion of cellulose during this period suggests that bacterial action was not extensive. However, Baker ('39) observed that hemicellulose and pectic substances are readily attacked by the iodophilic microflora of the rumen and bacterial action of this nature may be responsible for much of the carbohydrate digestion observed. Whether the microorganisms play a role in the metabolism of the protein which was so rapidly removed from the plant skeleton is not clear.

During the second 6 hours after feeding cellulose was rapidly disintegrated. The digestion of both proteins and carbohydrates paralleled the digestion of cellulose. Digestion of lignin in the rumen never exceeded more than a few per cent. The marked contrast between the fate of lignin in the rumen and in other parts of the digestive tract suggests that functionally the rumen is not a factor in the degradation of lignin. This point is particularly supported by the observation that although the fecal digestion of lignin in the hay fed cow R-4 was 32.7%, the digestion of lignin in the rumen 12 hours after feeding was zero. The rapid removal of cellulose during the second 6-hour period indicates that the cytoplasmic processes of the iodophilic microorganisms described by Baker ('42a) and observed by Bortree ('46) are likely to be the pre-

dominant phenomena throughout this period. By employing polariscopic techniques Baker ('39) and Bortree ('46) have observed that the contours of the area of disintegration of plant fragments removed from the rumen correspond precisely with the location of the microorganisms. This observation is highly indicative that the observed organisms are the effective agents of disintegration in the rumen. Rumen microorganisms are apparently capable of digesting widely varying amounts of roughage with equal efficiency since Hale, Duncan and Huffman ('40) did not observe any differences in the extent of rumen digestion of alfalfa hay when fed at levels varying from 10 to 30 pounds per day.

Rumen digestion was observed to come to a standstill within 12 hours after feeding and a maximum level of digestion was always obtained at this time. No significant increase in digestion coefficients was observed when the removal of rumen contents was delayed until 24 hours after feeding instead of 12 hours.

The marked decrease in rumen digestion during the second 12-hour period is in contrast to the high rate of digestion observed during the first 12-hour period. Rumen digestion coefficients for the various lots of hay used in these studies varied from 45.7 to 48.7% while fecal coefficients for the same lots of roughage varied from 46.7 to as much as 59.8%. Rumen coefficients thus appear to remain constant, irrespective of the fecal digestibility of the hay. This suggests a "ceiling" on rumen digestion, corresponding to the lowest level of fecal digestion usually observed in digestion trials. The probable role of lignin in imposing this "ceiling" on rumen digestion has been suggested by digestion trials at this station (Hale, Duncan and Huffman, '47). The fecal digestion of lignin was observed to vary from zero to as high as 47%. When the digestibility of lignin was zero, fecal digestion for dry matter corresponded to the average rumen digestion of dry matter. When fecal digestion of lignin was noted, the fecal digestion of dry matter was observed to exceed rumen digestion coefficients in direct ratio to the amount of lignin digested.

Evidence is thus obtained that the chemical composition of the plant material imposes certain limitations upon the activity of rumen microflora. The data suggest that lignin, which is relatively indigestible in the rumen, is important in imposing a limit on the rumen digestion of roughages. In this connection Baker and Martin ('37) and Baker ('39) reported that highly lignified tissues remained intact while slightly lignified tissues rich in hemicellulose were rapidly disorganized. Cutin and suberin were not attacked by the iodophile bacteria and therefore conferred immunity to the structure in which they were incorporated.

After the plant fragments pass from the rumen lignin is digested to a variable extent. This digestion is most likely produced by the acid content of the abomasum, since lignin is known to be partially soluble in dilute acid solution (Harris and Mitchell, '39), as well as some enzyme present in the gastric juice (Csonka, Phillips and Jones, '29). In any event the variable digestion of lignin after its passage from the rumen exposes cellulose and protein to further decomposition in the caecum. Under the conditions of these experiments an average of 11.6% cellulose and 9.5% protein was released in this manner after passing from the rumen. This digestion is most probably brought about by the iodophile microflora of the caecum and herein lies the major function of that organ in the ruminant animal. As this digestion is quite variable and usually not very extensive, the caecum can be considered to act only in a supplementary capacity to the rumen. Regarding the function of the caecum in ruminants Baker ('39) states, "Thus, whereas in the caecum of nonruminants the disintegration of cellulose is actively in progress, in the caecum of ruminants the changes taking place in the rumen have long since come to a standstill." He further observed that plant fragments in the caecum are in an advanced stage of disintegration but that the iodophile microorganisms so conspicuous upon them in the rumen are, for the most part, absent from the still visible areas of erosion.

In contrast to the rapid rate of rumen digestion reported in this paper, McAnally ('42) found that several days were required to obtain any considerable digestion in sacs suspended in the rumen. The causes of the impeded digestion are not altogether clear but it is probable that the lack of direct mastication, salivation and rumination was responsible. This suggests that these physical factors acting upon ingesta before it leaves the rumen serve to accelerate the rate of rumen digestion. Baker and Martin ('37) noted that gross fracture of the plant structures facilitated entrance of microorganisms. The degree to which such relationships are effective needs further clarification.

Rumen synthesis of fatty acids was demonstrated with both alfalfa hay and beet pulp rations. Since the net increases shown in table 5 do not include the increases resulting from the concentration of digestion, these values are considered especially significant. The observation that synthesized fat is rapidly dissipated from the rumen together with calculated increases approaching 300% indicates that fat synthesis may be sufficient to make a highly significant contribution to the nutrition of the ruminant. Whether or not the fatty acids observed in the rumen are intermediate or end products of digestion is not clear. Baker ('39) found that the iodophile microflora of the rumen not only digest cellulose but also act as acceptors for the products of digestion. He concluded provisionally (Baker, '42b) that materials synthesized in the rumen, such as polysaccharides, rather than the initial products of digestion, such as organic acids, are utilized by the host animal. Van der Wath and Myburgh ('41) are of the opinion that iodophile bacteria are able to synthesize glycogen within their bodies by utilizing the products of digestion. It may be, therefore, that the increases in fatty acids observed herein were due to an accumulation of the initial products of digestion not yet synthesized to polysaccharides by the microflora. Synthesis of the fatty acids to polysaccharides could further account for the rapid dissipation of the fatty acids from the rumen. On the other hand, the fatty acids may be utilized by



the animal as such or may represent products of bacterial metabolism. Controlled microbiological studies are necessary for the resolution of this problem. The nature of the fatty acids present in the rumen was not investigated.

#### SUMMARY

Studies of the chemical changes in the rumen suggest the following salient features regarding the fate of roughage in the ruminant digestive tract:

1. During the first 6 hours after feeding the predominant phenomenon was the rapid digestion of proteins and carbohydrates. Decomposition of cellulose began during this period but was not extensive.

2. The predominant phenomenon during the second 6 hours was the rapid disintegration of cellulose. Digestion of proteins and carbohydrates in this period paralleled the digestion of cellulose. Although small amounts of lignin may be attacked in the rumen, functionally the rumen is not a factor in lignin digestion.

3. Rumen digestion was observed to come to a standstill within 12 hours after feeding. Prolonging the digestion periods to 24 hours did not increase rumen digestion coefficients. Lignin apparently protects plant fragments from further action by rumen microflora.

4. Average rumen digestion coefficients for 8 trials at 12-14 hours after feeding were: dry matter 48.4, protein 59.6, nitrogen-free extract 65.2, crude fiber 27.2, cellulose 43.4, other carbohydrates 83.0 and lignin 3.1%.

5. After plant fragments pass from the rumen variable amounts of lignin may be digested, thereby exposing varying amounts of cellulose and protein to further digestion. Fecal digestion was observed to be quite variable even though rumen digestion of the same lots of hay was very constant.

6. The caecum plays only a supplementary role in the disintegration of roughage within the ruminant organism. An average of 11.6% of the cellulose and possibly 9.5% protein was observed to be digested in the caecum.

7. The production of fatty acids in the rumen was demonstrated. Maximum increases, after the increase due to digestion was eliminated, were 54.9% on an alfalfa hay ration and 281.7% on a beet pulp ration.

8. The rapid dissipation of fatty acids from the rumen together with the marked increases noted suggests that fatty acids may make a highly significant contribution to the nutrition of the ruminant either as an intermediate or end product of digestion.

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